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WO 99-61589

1/2

Date: 02 dec 1999

Destination: Agent

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PC

(51) International Patent Classification 6 : C12N 5/10, A61K 48/00 // C07K 14/705 — A2

(11) International Publication Number: WO 99/61

(43) International Publication Date: 2 December 1999 (02.12.

(21) International Application Number: PCT/US99/11825

(22) International Filing Date: 27 May 1999 (27.05.99)

(30) Priority Data:
60/086,988 28 May 1998 (28.05.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/086,988 (CIP)
Filed on 28 May 1998 (28.05.98)

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: EXPANSION OF HEMATOPOIETIC STEM CELLS TRANSDUCED WITH MDR-1 AND METHODS OF USE THEREOF

(57) Abstract

The present invention includes methods of performing *ex vivo* expansion of gene–modified hematopoietic stem cells which are useful for many applications involving bone marrow transplantation and *ex vivo* gene therapy. The present invention further includes the gene–modified hematopoietic stem cells that are used and produced by such methods. Such gene–modified hematopoietic stem cells can also contain a second heterologous gene. In addition, the present invention also includes methods of engrafting the gene–modified hematopoieteic stem cells of the present invention into animals, including for *ex vivo* gene therapy and for reconstitution of hematopoieteic cells in ablated mammals.

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EXPANSION OF HEMATOPOIETIC STEM CELLS TRANSDUCED WITH MDR-1 AND METHODS OF USE THEREOF

RESEARCH SUPPORT

The research leading to the present invention was supported in part by the National Institutes of Health Grant No: POl 53749-04. The government may have certain rights in the present invention. Support for this invention was also provided by the AMERICAN LEBANESE SYRIAN ASSOCIATED CHARITIES.

FIELD OF THE INVENTION

The present invention concerns methods of performing *ex vivo* expansion of gene-modified hematopoietic stem cells which are useful for many applications involving bone marrow transplantation and *ex vivo* gene therapy. The present invention also includes the gene-modified hematopoietic stem cells.

BACKGROUND OF THE INVENTION

Hematopoietic stem cells represent attractive targets for genetic modification since their progeny make up the entire spectrum of the hematopoietic system. Gene therapy involving stem cells is thus an expanding field that potentially has important applications in the treatment of a wide range of diseases [Nienhuis, AW, *et al.*, *Cancer*, **67**:2700 (1991)]. However, due to the inherent quiescent nature of stem cells, retroviral gene transfer is limited since stable integration requires cell division. Improved transduction of this target cell population is thus one of the major goals of current gene therapy research. In the mouse, gene transfer and repopulation with genetically-modified bone marrow stem cells following transplantation has been reported [Lemischka, IR, *et al.*, *Cell*, **45**:917 (1986) and Dick, *et al.*, *Cell*, **42**:71 (1985)]. Whereas the level of stem cell gene transfer and expression are relatively modest, it has been sufficient to investigate effects of gene expression on hematopoiesis [Persons, *et al.*, *Blood 90:1777* (1997)]. In humans, only an extremely

low number of transgenic stem cells persist on a long-term basis [Brenner, *et al.*, *Lancet*, **342**:1134 (1993) and Brenner, *et al.*, *Lancet*, **341**:85 (1993)] and Rill, *et al.*, *Blood*, **84**:380 (1994)]. Therefore there is a need for increasing the proportion of such transdueed stem cells through *ex vivo* expansion following transduction and/or through *in vivo* selection approaches.

Most current protocols for transduction of stem cells employ *in vitro* liquid suspension culture with hematopoietic growth factors. It is now well established that culturing murine bone marrow cells for 4 days in the presence of defined concentrations of interleukin-3, interleukin-6, and stem cell factor does not adversely effect overall stem cell survival and function. However, expansion beyond this point has not proven to be beneficial and results in depletion of the reconstitution potential of the bone marrow graft. Cytokine-stimulated stem cells cultured in expansion conditions typically either undergo differentiation or programmed cell death (apoptosis). More mature populations such as the CFU-S and CFU-C, however, are capable of significant expansion in culture. However, these cells are distinct from stem cells and only provide short to moderate-term repopulating ability in transplanted mice. In humans, the long-term culture-initiating cell (LTC-IC) can be expanded *in vitro* with appropriate combinations and amounts of growth factors. LTC-ICs have recently been shown to be more mature than the SCID mouse repopulating cell (SRC) [Dick *et al.*, *Cell* **42**:71 (1985)]. SRCs are depleted in cultures that are more than 4 days old, which is consistent with the SRC being a more primitive cell type. More recently, culturing hematopoietic stem cells derived from the AGM (a pre-liver intraembryonic site) has been reported [Dzierzak *et al.* WO 98/12304, hereby incorporated by reference in its entirety]. However, the prior art teaches at most a four-fold expansion of human hematopoietic cells [Bhatia *et al.*, J. Exp. Med., **186**:619-624 (1997)].

Methodology for enriching pluripotent stem cells in culture could have a major impact on treatment of blood and immune-system disorders. For example, bone marrow transplantation is often the only option for persons having hematopoietic and immune-

system dysfunctions caused by congenital disorders and/or chemotherapy or radiation therapy. In addition, enriching pluripotent stem cells should greatly enhance the treatment of immunodeficency disorders. Furthermore, the effectiveness of the treatment of blood diseases by *ex vivo* gene therapy, *e.g.*, treating sickle cell anemia or thalassemia, could also be substantially enhanced. Therefore, expansion of primitive stem cells in culture would be a major advance for all aspects of bone marrow transplantation as well as gene therapy applications. Unfortunately, despite the clear need for such methodology, heretofore, it has not been realized.

The citation of any reference herein should not be deemed as an admission that such reference is available as prior art to the instant invention.

SUMMARY OF THE INVENTION

One important aspect of the present invention is a method of performing *ex vivo* expansion of a gene-modified hematopoietic stem cell. One embodiment of this type comprises transducing a hematopoietic stem cell with a nucleic acid encoding a transmembrane efflux pump and then culturing the transduced cell, *i.e.* the gene-modified hematopoietic stem cell, *ex vivo*, thereby expanding the gene-modified hematopoietic stem cell. In a preferred embodiment, the gene-modified hematopoietic stem cell is expanded at least 10-fold.

As exemplified below in one embodiment the transmembrane efflux pump is human multidrug resistance-1 (*i.e.*, MDR-1, the P-glycoprotein) which is encoded by the nucleotide sequence of SEQ ID NO:1 and has the amino acid sequence of SEQ ID NO:2 . In another embodiment the transmembrane efflux pump is murine MDR-1 which is encoded by the nucleotide sequence of SEQ ID NO:5 and has the amino acid sequence of SEQ ID NO:6 . In yet another embodiment the transmembrane efflux pump is murine MDR-3 which is encoded by the nucleotide sequence of SEQ ID NO:7 and has an amino acid sequence of SEQ ID NO:8. In a particular embodiment inhibitors of P-glycoprotein, such as PSC833, are added to the expansion culture. In

yet another embodiment the transmembrane efflux pump is MRP (multidrug resistant protein). In still another embodiment, the transmembrane efflux pump is the cystic fibrosis membrane transporter.

In one embodiment, the method comprises culturing the gene-modified hematopoietic cell in the presence of one or more cytokines. In one such embodiment the culture contains 5 to 300 ng/ml of the cytokine. In a particular embodiment the culture contains 10 to 50 ng/ml of the cytokine. In another embodiment the culture contains 0.5 to 10 ng/ml of the cytokine.

In one embodiment the cytokine is an early-acting hematopoietic cytokine. In a particular embodiment the cytokine is interleukin-3. In another embodiment the cytokine is interleukin-6. In still another embodiment the cytokine is stem cell factor. In still another embodiment the cytokine is G-CSF. In yet another embodiment the cytokine is GM-CSF. In still another embodiment the cytokine is the FLT-3 ligand. In yet another embodiment the cytokine is interleukin-1. In still another embodiment more than one of these cytokines are present. In a particular embodiment interleukin-3, interleukin-6, and stem cell factor are all present.

In another embodiment of the method of performing *ex vivo* expansion of a gene-modified hematopoietic stem cell, the cell is expanded for at least 3 days. In alternative embodiment the gene-modified hematopoietic stem cell is expanded for at least 6 days. In a particular embodiment the gene-modified hematopoietic stem cell is expanded for at least 9 days. In a preferred embodiment the gene-modified hematopoietic stem cell is expanded for at least 12 days. In a related embodiment the gene-modified hematopoietic stem cell further comprises a second heterologous gene.

In still another embodiment of the method of performing *ex vivo* expansion of a gene-modified hematopoietic stem cell, the hematopoietic stem cell is a mammalian hematopoietic stem cell. In a particular embodiment the mammalian hematopoietic stem cell is a murine hematopoietic stem cell. In a preferred embodiment the

mammalian hematopoietic stem cell is a human hematopoietic stem cell. In another preferred embodiment the gene-modified hematopoietic stem cell expresses a splice-corrected version of the human MDR-1, as exemplified below.

In another embodiment the method comprises transducing the hematopoietic stem cell with a viral vector that comprises a nucleic acid encoding a transmembrane efflux pump. In a particular embodiment the transmembrane efflux pump is MDR-1. In one such embodiment the viral vector is a herpes simplex viral vector. In another embodiment the viral vector is an adenoviral vector. In still another embodiment the viral vector is an adeno-associated viral vector (AAV). In a particular embodiment the viral vector is a defective virus, preferably not encoding a gene for a functional viral protein.

In an alternative embodiment of the method, the viral vector is a retroviral vector. In one such embodiment the retroviral vector is an HIV retroviral vector. In another embodiment the vector is a VL 30 vector. In yet another embodiment the vector is a MSCV retroviral vector. As exemplified below the retroviral vector can be a Harvey Murine Sarcoma Vector. In one such embodiment the hematopoietic stem cell is transduced by being co-cultured with a retroviral producer cell line. In still another embodiment of the method, transducing the hematopoietic stem cell with a transmembrane efflux pump, *e.g.*, MDR-1, is performed with a DNA vector (*i.e.*, a naked DNA) that comprises a nucleic acid encoding the MDR-1.

In a particular embodiment the nucleic acid encoding a transmembrane efflux pump, *e.g.*, MDR-1, is introduced into the hematopoietic stem cell with a non-integrating vector *e.g.*, an adenoviral vector. Such an adenoviral vector would only be expressed transiently, during the period of *in vitro* expansion. This contrasts with the retroviral vector exemplified below which is integrated and expressed continuously *in vivo*. In another embodiment, the nucleic acid encoding MDR-1 is introduced into the hematopoietic stem cell with Murine Stem Cell Virus which lacks the VL30

sequences in the Harvey Murine Sarcoma vector [Hawky *et al.*, *Gene Therapy* 1:136 (1994)].

The present invention further provides a gene-modified hematopoietic stem cell that has been transduced with a nucleic acid encoding a transmembrane efflux pump, *e.g.*, MDR-1 and has been expanded. In one embodiment the hematopoietic stem cell is a mammalian hematopoietic stem cell. In a particular embodiment the hematopoietic stem cell is a murine hematopoietic stem cell. In a preferred embodiment the mammalian hematopoietic stem cell is a human hematopoietic stem cell. In another preferred embodiment the gene-modified hematopoietic stem cell expresses a splice-corrected version of the human MDR-1 as exemplified below.

In one embodiment the gene-modified hematopoietic stem cell has been expanded for at least 3 days. In another embodiment the gene-modified hematopoietic stem cell has been expanded for at least 6 days. In a particular embodiment the gene-modified hematopoietic stem cell has been expanded for at least 9 days. In a preferred embodiment the gene-modified hematopoietic stem cell of has been expanded for at least 12 days. In a particular embodiment of this type the gene-modified hematopoietic stem cell has been expanded for 16 days or more. In a related embodiment the gene-modified hematopoietic stem cell further comprises a second heterologous gene.

Methods of engrafting an animal with the gene-modified hematopoietic stem cell of the present invention are also provided. Preferably, the gene-modified hematopoietic stem cell has been expanded as taught herein. One embodiment comprises placing the expanded gene-modified hematopoietic stem cell into an animal. In one such method, placing the cell into the animal is performed by injection. In a particular embodiment more than one injection is made. In another embodiment multiple injections are made over the course of several days (*e.g.*, in humans 1 to 20 days appears to be a reasonable range). In one embodiment the animal is a mammal. In a particular embodiment the mammal is a mouse. In a preferred embodiment the mammal is a

human. Preferably the engrafted cell is stable for at least three months, and more preferably six months, or a year or even longer.

The present invention further provides methods of treating an animal in need of treatment for a hematopoietic stem cell deficiency using a method of engrafting of the present invention. In one embodiment of this method the hematopoietic stem cell is transduced *ex vivo* with a nucleic acid encoding a transmembrane efflux pump, *e.g.* MDR-1. The transduced hematopoietic stem cell (a gene-modified hematopoietic stem cell) is expanded and then engrafted into the animal. In a preferred embodiment, the hematopoietic stem cell is obtained from the animal in need of treatment, and then after being transduced with a nucleic acid encoding MDR-1 and expanded, the resulting gene-modified hematopoietic stem cell is placed back into the animal. In a particular embodiment the animal is a mammal. In a preferred embodiment of this type, the mammal is a human.

A gene-modified hematopoietic stem cell used in a method of engrafting an animal of the present invention can further comprise a second heterologous gene. Such methods include *ex vivo* gene therapy which may be used to treat diseases involving a dysfunctional cell that is derived from an hematopoietic stem cell. Thus, any genetic defect that could be corrected by bone marrow transplantation can be treated by the methods described herein. In one such embodiment, the second heterologous gene encodes a functional β-globin. In another embodiment, the second heterologous gene encodes a functional adenosine deaminase. In still another embodiment, the second heterologous gene encodes a functional glucocerebrosidase.

Accordingly, it is a principal object of the present invention to provide a method of expanding hematopoietic stem cells *ex vivo.*

It is a further object of the present invention to provide an expanded hematopoietic stem cell.

It is a further object of the present invention to provide a method for reconstituting bone marrow cells in an animal subject after the animal has undergone chemotherapy or radiation therapy.

It is a further object of the present invention to provide a method for providing bone marrow cells for a human subject while the human is undergoing chemotherapy.

It is a further object of the present invention to provide a method of engrafting a gene-modified hematopoietic stem cell into an animal subject.

It is a further object of the present invention to provide a method of performing *ex vivo* gene therapy on an animal subject by engrafting an expanded gene-modified hematopoietic stem cell into the animal subject wherein the gene-modified hematopoietic stem cell further comprises a therapeutic gene.

These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1B show the expansion kinetics for total cells (Figure 1A) and drug-resistant progenitors (Figure 1B) following retroviral transduction. Cells were maintained in liquid suspension cultures with addition of murine IL-3, human IL-6, and rat SCF. Figure 1A shows a typical cell expansion for cells from either MDR-1 or DHFR co-cultures. No significant difference in cell expansion was noted between these groups. Cells were removed at 6 day intervals and assayed for clonogenic progenitors in methylcellulose. Selective concentrations of taxol or trimetrexate were used to determine MDR-1 or DHFR expressing progenitor cells respectively. Figure 1B shows the drug-resistant progenitor population was found to expand extensively and typically reached 100-fold by 2 weeks.

Figures 2A-2B show the long-term analysis of engraftment with donor bone marrow in non-irradiated recipients. HW80 recipient mice were injected for 5 consecutive days with transduced bone marrow cells (C57) which had been expanded in culture for 12 to 16 days. Later the same day, mice were treated with trimetrexate (130 mg/kg) and NBMPR-P (20 mg/kg). Beginning at 1 week post-transplant, donor C57 hemoglobin levels were quantitated by electrophoresis on cellulose acetate gels. Persistent engraftment was only seen in mice receiving expanded bone marrow cells transduced with HaMDR-1 (5/12) as shown in Figure 2B. Engrafted mice included: MDR 7 (■), MDR 11 (▲), MDR 18 (◆), MDR 20 (▼) and from the second experiment MDR 15 (●). No stable engraftment was seen in mock-transduced (0/8) or DHFR-transduced (0/8) expanded bone marrow as shown in Figure 2A. Shown in Figures 2A-2B are mice from 2 independent expansion experiments.

Figures 3A-3C show representative hemoglobin electrophoresis gels from non-irradiated mice engrafted with expanded bone marrow (5 months post-BMT for MDR 20 and 7 months post-BMT for all others). C57 bone marrow was used as the donor marrow for transduction and expansion. Recipient mice were HW80. The differing hemoglobin patterns are indicated in Figure 3A. Primary recipients shown in Figure 3B are MDR 7, 11, 18, 20 from expt. #1 and MDR 15 from expt. #2. Secondary irradiated recipients were transplanted with marrow cells from MDR 20 demonstrating persistence of donor engraftment following secondary transplant, (a formal proof of stem cell engraftment) as shown in Figure 3C. Figure 3D shows the FACS analysis for P-glycoprotein (Pgp) expression in red blood cells from engrafted mice. Peripheral red blood cells were stained with a monoclonal antibody to human Pgp followed by FACS analysis for the PE chromophore. As a negative control, a mouse injected with HaDHFR-transduced marrow (DHFR #1) is shown. All 4 mice from expt. #2 analyzed at 10 weeks post-transplant demonstrate significant levels of Pgp positive red blood cells. When corrected for the percent donor red cells present at the time of analysis, levels of Pgp positivity approached 100% of circulating donor red blood cells.

Figures 4A-4C show the competitive repopulation assay to determine the relative stem cell content of *ex vivo* expanded bone marrow versus fresh non-expanded marrow. C57 donor bone marrow cells were transduced with HaMDR-1. HW80 donor bone marrow cells were transduced with HaDHFR. Cells were expanded for 12 days in culture then combined according to hind limb volume. 0.005 vols. expanded cells were competed against 0.25 vols. of fresh competing marrow. MDR-1 expanded cells effectively competed against fresh HW80 marrow (Figure 4A, left). DHFR-expanded marrow was completely out competed by fresh C57 marrow (Figure 4A, middle). When MDR-1 (C57) was competed against DHFR (HW80) at equal vols. mice reconstituted solely with MDR-1 marrow, indicating a much greater stem cell content (Figure 4A, right). The comparison of the bands for the distinctive Hb patterns of C57 and HW80 are shown in Figure 4B. Figure 4C shows the Hb patterns in recipient mice (lanes 1-10) as analyzed by Hb electrophoresis.

Figure 5 shows the secondary CFU-S analysis for HaMDR-1 marked primitive hematopoietic cells. At time points from 10 to 24 weeks post-transplant, primary recipients from MDR vs. DHFR competitive repopulation mice (n=6) and MDR 15 were sacrificed and bone marrow cells were injected into secondary recipients. Day 12 CFU-S were harvested and DNA was prepared for Southern blot analysis. DNA was restricted with EcoR1 and probed with an MDR-1 specific probe. A band of the correct size was seen in all CFU-S examined (88/88) from 7 individual mice. 56 representative examples are shown. Negative controls included CFU-S from mice transplanted with untransduced bone marrow. All 10 of these (4 shown) did not contain HaMDR-1 retroviral DNA.

Figure 6A shows the kinetic analysis of HaMDR-1 transduced stem cell expansion. Bone marrow cells were transduced with the HaMDR-1 retrovirus and expanded for the indicated time points. HaMDR cells (0.02 vols/HW80 background) were combined with fresh competed marrow (0.25 vols/C57 background) and injected into lethally irradiated mice. Unexpanded MDR-1 bone marrow (day 0) did not out compete fresh marrow. However, expansion for 3 to 12 days resulted in a progressive

increase in engraftment. Figure 6B shows Southern blot analysis of hemoglobin DNA for multilineage engraftment. DNA was prepared from the peripheral blood of mice #20, 21, and 26. DNA was restricted with EcoR1 and probed with a hemoglobin-specific probe. The appropriate bands are indicated by an arrow for both C57 (single) and HW80 (diffuse). The level of lympho-myeloid engraftment determined by DNA analysis correlates very well with the level of engraftment determined in the erythroid lineage by hemoglobin electrophoresis.

Figure 7A shows the kinetics of white blood cell elevation in mice engrafted with expanded bone marrow. The WBC count for all 10 engrafted mice from Figures 6A-6B are shown as they were examined serially. Mice typically had a long latent period of above normal to normal WBC counts followed by a rapid phase observed from 5 to 8 months later. Two mice from this experiment maintain long-term engraftment and continue to have normal WBC counts at time points 8.5 months post-transplant. Figure 7B shows Wright-stained peripheral blood smears from mice displaying an abnormal cell population. A normal mouse smear at the indicated magnifications is shown (top). The second example demonstrates the most common morphology seen (middle). In a few rare cases, the third blast-like phenotype was seen indicating probable transformation from a myeloproliferative disorder into a leukemia (bottom).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods of performing *ex vivo* expansion of gene-modified hematopoietic stem cells which are useful for many applications including bone marrow transplantation, and *ex vivo* gene therapy. In addition, the present invention provides methods of engrafting the gene-modified hematopoietic stem cells of the present invention into animals, including for bone marrow transplantation and *ex vivo* gene therapy. Therefore the present invention provides methods of treating an animal in need of treatment for a hematopoietic stem cell deficiency using a method of engrafting the expanded gene modified hematopoietic stem cells of the present invention. In one such embodiment the hematopoietic stem

cell is transduced *ex vivo* with a nucleic acid encoding a transmembrane efflux pump, *e.g.* MDR-1. The transduced hematopoietic stem cell (*i.e.*, a gene-modified hematopoietic stem cell) is expanded and then engrafted into the animal. Preferably the hematopoietic stem cell used is matched with the recipient animal to minimize and/or prevent host rejection. Thus, the hematopoietic stem cell is preferably obtained from the animal in need of treatment, and then after being transduced with a nucleic acid encoding MDR-1 and expanded, the resulting gene-modified hematopoietic stem cell is placed back into the animal. The treatments as described herein may be used for any hematopoietic stem cell deficiency including that due to radiation therapy and/or chemotherapy, *e.g.*, as used in cancer treatments. One particular advantage for treating a hematopoietic stem cell deficiency due to chemotherapy with a method of the present invention is that a gene-modified hematopoietic stem cell transduced with a nucleic acid encoding MDR-1 will also be protected from the chemotherapeutic and its adverse effects. Therefore, the engrafting of the gene-modified hematopoietic stem cell into the recipient animal can be performed concomitantly with the chemotherapy.

The *ex vivo* gene therapy methodology of the present invention can be used for treating any disorder (particularly a genetic disorder) that involves a defect in a cell derived from a hematopoietic stem cell including but not limited to the treatment of thalassemia (*e.g.*, with an expanded modified hematopoietic stem cell encoding human β-globin), Gaucher's disease (*e.g.*, with an expanded modified hematopoietic stem cell encoding glucocerebrosidase), sickle cell anemia, and leukemia.

The present invention further provides the expanded gene-modified hematopoietic stem cells used and/or produced by such methods. Such expanded gene-modified hematopoietic stem cells can also contain a second heterologous gene.

In addition, the ability of expanding the otherwise rare hematopoietic stem cells provided by the present invention provides a source of hematopoietic stem cells which is large enough in quantity to allow standard biochemical analysis to be performed on this relatively unstudied cell type. Indeed, the present invention results

in the capability of the expanding of human hematopoietic stem cells that is greatly increased from the maximum of about four-fold expansion taught in the prior art. Thus, the present invention provides a means for performing facile assays for identifying factors involved in the regulation of the proliferation *versus* differentiation of hematopoietic stem cells, particularly human hematopoietic stem cells. Such assays, for example, can be based on the experimental conditions taught herein and the administration of fractionated cellular extracts. Naturally occurring factors can be identified by such assays and then isolated by convention biochemical procedures. Alternatively, chemical libraries and/or phage libraries can be used in an analogous drug screening assays. These naturally occurring factors and drugs can then be used to manipulate the fate of hematopoietic stem cells initially *in vitro* and eventually *in vivo*. Indeed, currently there are no known factors which specifically lead to the proliferation of hematopoietic stem cells in the absence of differentiation.

In Example 1, below, bone marrow cells were transduced with a Harvey (Ha)/MDR-1 retrovirus and expanded for 12 days in the presence of interleukin (IL)-3, IL-6, and stem cell factor (SCF). Long-term engraftment in non-irradiated mice was observed after transplantation of the cells which were transduced with Ha/MDR-1. To compare relative repopulating activities of expanded vs. unexpanded cells, competitive repopulation experiments in irradiated recipients were performed. These results showed at least a 10-fold increase in the absolute number of repopulating cells relative to fresh untransduced marrow. The results demonstrate that MDR-1 overexpression allows dramatic cytokine-driven expansion of hematopoietic stem cells *in vitro*.

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "hematopoietic stem cell" is a pluripotent cell that is able to either replicate itself with self-renewal divisions or to differentiate along a number of pathways and thereby generate erythrocytes, granulocytes, monocytes, mast cells, lymphocytes, and

megakaryocytes. These stem cells occur with a frequency of about 1 stem cell per 10^4 bone marrow cells.

A "heterologous gene" as used herein is a gene that is introduced into a hematopoietic stem cell through a molecular biological manipulation. As defined herein, this molecular biological manipulation is made such that the heterologous gene is inserted into the hematopoietic stem cell. The heterologous gene need not be expressed in the stem cell as long as it is expressed in the progeny of the stem cell. The coding sequence of the heterologous gene is operatively linked to an expression control sequence. Generally a heterologous gene is first placed into a vector. The heterologous gene is not necessarily naturally contained by the vector, though a heterologous gene can encode a protein that is native to a hematopoietic stem cell. For example, the heterologous gene can encode a functional protein and be used in *ex vivo* gene therapy to replace the corresponding defective gene in an hematopoietic stem cell. The heterologous gene will usually be flanked by DNA that does not flank the genomic DNA in the genome of the source organism. Alternatively, the heterologous gene may not be naturally found in the hematopoietic stem cell such as the gene for human MDR-1 introduced into a murine hematopoietic stem cell.

A cell has been "transduced" by a heterologous gene such as the MDR-1 gene (*i.e.*, a nucleic acid encoding MDR-1), when the gene has been introduced inside the cell and the coding sequence of the gene is operatively linked to an expression control sequence. The transducing gene is carried by a vector and the gene may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. A stably transduced cell is one in which the transducing gene has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the cell to establish cell lines or clones comprised of a population of daughter cells containing the transducing gene. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

As used herein a "gene-modified hematopoietic stem cell" is a hematopoietic stem cell that has been transduced by a heterologous gene. A gene-modified hematopoietic stem cell transduced with a nucleic acid encoding MDR-1 (the MDR-1 gene) is exemplified below.

As used herein the "expansion" of an hematopoietic stem cell indicates that there is an increase in the absolute number of hematopoietic stem cells, *i.e.*, during the culturing of the cells. Analogously, an hematopoietic cell that has undergone such expansion has been "expanded".

As used herein "engrafting" a hematopoietic stem cell, preferably an expanded hematopoietic stem cell, means placing the hematopoietic stem cell into an animal, *e.g.*, by injection, wherein the stem cell persists *in vivo*. This can be readily measured by the ability of the hematopoietic stem cell to contribute to the ongoing blood formation.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; *i.e.*, capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. The term "vector" can also refer to a recombinant virus or defective virus containing a replicon to which another DNA segment may be attached.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-

stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

A "coding sequence" is a nucleic acid sequence which can be reverse transcribed (*i.e.*, when part of a retroviral vector) and/or transcribed and then translated into a polypeptide *in vitro* and/or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

A nucleic acid sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and/or translation of that nucleic acid sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the nucleic acid sequence to be expressed and maintaining the correct reading frame to permit expression of the nucleic acid sequence under the control of the expression control sequence and production of the desired product encoded by the nucleic acid sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal for example, such a start signal can be inserted in front of the gene.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

Any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma, adenovirus, herpes virus and other sequences known to control the expression of genes of mammalian cells, and various combinations thereof.

In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential secondary structures. Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence combinations that will express the heterologous genes used in the present invention.

A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes. For instance, alpha-factor, a native yeast protein, is secreted from yeast, and its signal sequence can be attached to heterologous proteins to be secreted into the media (See U.S. Patent 4,546,082, EPO 0 116 201, publication date 12 January 1983; U.S. Patent Application Serial No. 522,909, filed 12 August 1983). Further, the alpha-factor leader and its analogs have been found to secrete heterologous proteins from a variety of yeast, such as Saccharomyces and Kluyveromyces, (EPO 88312306.9 filed 23 December 1988; U.S. Patent Application Serial No. 139,682. filed 30 December 1987, and EPO Publication No. 0 301 669, publication date 1 February 1989).

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15 percent, preferably by at least 50 percent,

more preferably by at least 90 percent, and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host.

Vectors

According to the present invention, the vector for *ex vivo* administration of the gene encoding MDR-1 (*i.e.*, a nucleic acid encoding MDR-1) and/or an alternative heterologous gene can be introduced via any strategy. Vectors can be introduced to transduce the desired host cells *ex vivo* by methods known in the art, *e.g.*, transfection, electroporation, microinjection, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, using a viral vector, with a DNA vector transporter, and the like. Alternatively, the vector can be introduced by lipofection.

Viral vectors are commonly used for *ex vivo* targeting and therapy procedures; these include DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art [*see, e.g.*, Miller and Rosman, *BioTechniques* 7:980-990 (1992)]. DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. In addition, different viral vectors may exhibit specificity for one or another cell type. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector [Kaplitt et al., *Molec. Cell. Neurosci.* 2:320-330 (1991)], defective herpes virus vector lacking a glyco-protein L gene [Patent Publication RD 371005 a], or other defective herpes virus vectors [International Patent Publication No. WO 94/21807, published September 29, 1994; International Patent Publication No. WO 92/05263, published April 2, 1994]; an attenuated adenovirus vector, such as the vector described by

Stratford-Perricaudet et al. [*J. Clin. Invest.* **90**:626-630 (1992); *see also* La Salle et al., *Science* **259**:988-990 (1993)]; and a defective adeno-associated virus vector [Samulski et al., *J. Virol.* **61**:3096-3101 (1987); Samulski et al., *J. Virol.* **63**:3822-3828 (1989); Lebkowski et al., *Mol. Cell. Biol.* **8**:3988-3996 (1988)]. Herpes virus vectors are preferred for dendritic cells.

In another embodiment, the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson et al., U.S. Patent No. 5,399,346; Mann et al., 1983, Cell 33:153; Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., 1988, J. Virol. 62:1120; Temin et al., U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty et al.; and Kuo et al., 1993, Blood 82:845. International Patent Publication No. WO 95/07358 describes high efficiency transduction of primary B lymphocytes. In a specific embodiment, exemplified below, a Harvey murine retroviral vector is used to transduce hematopoietic stem cells. Retroviral vectors can be constructed to function as infectious particles or to undergo a single round of transfection. In the former case, the virus is modified to retain only those genes responsible for packaging and replication and to express the heterologous gene. Non-infectious viral vectors are prepared to destroy the viral packaging signal, but retain the structural genes required to package the co-introduced virus engineered to contain the heterologous gene and the packaging signals. Thus, the viral particles that are produced are not capable of producing additional virus.

For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* or *ex vivo* transfection of a gene encoding a marker [Felgner, et. al., *Proc. Natl. Acad. Sci. U.S.A.* **84**:7413-7417 (1987); *see* Mackey, et al., *Proc. Natl. Acad. Sci. U.S.A.* **85**:8027-8031 (1988); Ulmer et al., *Science* **259**:1745-1748 (1993)]. The use of cationic lipids may promote encapsulation of

negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes [Felgner and Ringold, *Science* **337**:387-388 (1989)].

It is also possible to introduce the vector as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter [*see, e.g.*, Wu et al., *J. Biol. Chem.* **267**:963-967 (1992); Wu and Wu, *J. Biol. Chem.* **263**:14621-14624 (1988); Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990; Williams et al., *Proc. Natl. Acad. Sci. USA* **88**:2726-2730 (1991)].

The present invention includes vectors containing a gene (i.e., nucleic acid) encoding a transmembrane efflux pump, *e.g.*, MDR-1. Also included are truncated forms, analogs and derivatives of the transmembrane efflux pump, *e.g.*, MDR-1 that have essentially the same or improved functional activity as MDR-1, for example. Therefore, the production and use of derivatives and analogs related to MDR-1, for example are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type MDR-1 protein.

In particular, MDR-1 derivatives, for example, can be made by altering encoding nucleic acid sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced or increased functional activity as it relates to the present invention, relative to the native MDR-1, for example.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a MDR-1 gene, for example, may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of MDR-1 genes which are altered

by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, the MDR-1 derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a MDR-1 protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Additionally, the nucleic acid sequence encoding a transmembrane efflux pump, such as MDR-1, can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional activity of the mutated MDR-1 gene product, for example. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479-488; Oliphant et al., 1986, Gene 44:177; Hutchinson et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710), use of TAB® linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis (see Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70).

Expression vectors containing a transmembrane efflux pump gene, such as the MDR-1 gene, inserts can be identified by many ways including : (a) PCR amplification of the desired plasmid DNA or specific mRNA, (b) nucleic acid hybridization, (c) presence or absence of "marker" gene functions, and (d) expression of inserted sequences. In the first approach, the nucleic acids can be amplified by PCR with incorporation of radionucleotides or stained with ethidium bromide to provide for detection of the amplified product. In the second approach, the presence of a MDR-1 gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted MDR-1 gene. In the third approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, β-galactosidase activity, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. In the fourth approach, recombinant expression vectors can be identified by assaying for the activity of the MDR-1 gene product expressed by the recombinant.

Promoters

According to the present invention, the gene encoding MDR-1 and/or a second heterologous gene can be under the control of any promoter. In a specific embodiment, the human cytomegalovirus (CMV) immediate early promoter is used to effect transient expression of MDR-1. Alternatively, an inducible promoter can be used. However, the present invention contemplates use of any promoter to control expression of MDR-1. Selection of the promoter depends on the desired use. For example, expression of MDR-1 may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host or host cell selected for expression. Promoters which may be used to control MDR-1 gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the

herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); and using a transcriptional control region such as the beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94). Alternatively, expression of the MDR-1 gene can be under control of an inducible promoter, such as metallothionein promoter, which is induced by exposure to heavy metals.

Bone marrow transduction

Bone marrow cells can be obtained from any number of sources from an animal, including a human subject. For example, the cells can be harvested from iliac bone marrow. Alternatively, hematopoietic stem cells can be obtained from umbilical chord cells. Another source for hematopoietic stem cells is from circulating fetal blood cells. In addition, a human subject, for example, can be treated with a cytotoxic drug and/or a hematopoietic stem cell stimulating cytokine (*e.g.*, G-CSF). Mononuclear cells can then be collected by leukophoresis and the hematopoietic stem cells can be isolated from the peripheral blood cells by their selective binding to an antibody raised against CD34.

In the example below, bone marrow cells were flushed from the hind limbs of a laboratory animal and prestimulated for 48 hours in an appropriate medium. Dulbecco's modified essential medium supplemented with 15% fetal bovine serum, 100 units/ml penicillin, and 100 ng/ml streptomycin (P/S; Gibco-BRL) was also used in the Example below. Growth factors can also be included in the suspension culture at the appropriate concentration. Following prestimulation cells can be co-cultured on irradiated retroviral producer cell lines (e.g., ecotropic producer cell lines for mice and amphotropic producer cell lines for humans) for 48 hours in the presence of the same growth factor combination but also with added 6 mg/ml polybrene (Sigma) to enhance transduction.

Ex vivo culture and expansion of myeloid progenitors

After transduction, cells can be cultured. In the Example below, culturing was performed in the presence of interleukin-3, interleukin-6, and stem cell factor. Any other cytokine which supports the proliferation of hematopoietic stem cells could be used, including but not limited to GM-CSF, G-CSF and FLT-3 ligand. It is preferred that when the hematopoietic stem cell is a human cell and/or the recipient is a human subject that the cytokines used also be the human homolog.

Cells are typically resuspended at 1 x 10^6 cells/ml every 3-6 days for at least 12 days of expansion. Aliquots of cells can be removed for CFU-C analysis at various time points. The percentage of drug-resistant progenitors can calculated by plating cells in methylcellulose (Stem Cell Technologies) for example, in the presence of selective concentrations of drugs. The percentage of transduced cells are generally found to remain constant throughout expansion.

Non-irradiated recipient bone marrow transplants

For bone marrow transplants into non-irradiated recipients, mice can receive from 1 to 10 daily intravenous injections with a vector containing a nucleic acid encoding MDR-1, *e.g.*, a total of 20 - 40 x 10^6 cells for the 5 day period exemplified below. (Humans can receive from 1 to 20 such daily intravenous injections, preferably 5 to 10 daily intravenous injections). Following a five day treatment course in Example 1 below, the presence of a donor marker protein, Hb as exemplified below, can be monitored in recipient animal (as exemplified below the monitoring began at one week after the last injection), and then followed for as long as appropriate, (8 to 14 months in Example 1).

The present invention may be better understood by reference to the following non-limiting Example, which is provided as exemplary of the invention. This example is presented in order to more fully illustrate the preferred embodiments of the invention.

It should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1

EX VIVO EXPANSION OF MURINE HEMATOPOIETIC STEM CELLS TRANSDUCED WITH AN MDR-1 RETROVIRAL VECTOR

Introduction

Initial studies were intended to determine whether gene-modified progenitors could be expanded in culture and whether these relatively mature transduced cells would be useful for chemoprotection against myelosuppression in mice receiving antifolate chemotherapy. This progenitor approach previously has been shown effective with vectors expressing methyguanine DNA methyltransferase [MGMT] and protective against 1,3-bis(2-chloroethly)-1-nitrosourea [BCNU] mediated delayed myelosuppression. Unexpectedly, mice receiving bone marrow transduced with an MDR-1 retrovirus showed sustained engraftment in non-irradiated recipients. In addition, subsequent quantitation of the stem cell content by competitive repopulation experiments in lethally-irradiated mice indicated a large increase in the repopulation potential with expanded MDR-1 marrow. Such results were surprising since there had been numerous observations of negative effects of the expansion of stem cells when alternative gene-modified progenitors had been generated. A myeloproliferative disorder did result in some cases, since a fraction of mice engrafted with MDR-1 marrow developed a myeloproliferative disorder characterized by high peripheral white blood cell counts and splenomegaly. This disorder, however, was not found to be absolutely linked to the stem cell expansion described herein.

Methods

Retroviral producer cell lines and vector constructs: The Harvey (Ha)MDR-1 and HaDHFRL22Y vectors and ecotropic producer cell lines were generated as described previously [Sorrentino *et al.*, Science **257**: 99-103 (1992); Galipeau *et al.*, *Human*

Gene Therapy 8:1771-1783 (1997), hereby incorporated by reference in their entireties]. The MDR-1 protein (encoded by SEQ ID NO:1 and having the amino acid sequence of SEQ ID NO:2) contains a wild-type glycine 185 amino acid. Wild-type MDR-1 shows increased resistance to etoposide and decreased resistance to vinca alkaloids when compared with the valine 185 mutant (encoded by SEQ ID NO:3 and having the amino acid sequence of SEQ ID NO:4). The MDR-1 cDNA has been previously splice-corrected to allow for optimal levels of active protein expression in transduced cells [Galipeau *et al.*, *Human Gene Therapy* 8:1771-1783 (1997)]. The DHFRL22Y protein contains a leucine to tyrosine mutation at codon 22 (L22Y) which greatly optimizes resistance to trimetrexate [Spencer *et al.*, *Blood* 87:2579-2587 (1996)].

Retroviral-mediated bone marrow transduction: Bone marrow cells were flushed from the hind limbs of either C57/Bl6 (C57) or B6.C-H1/BY (HW80) congenic mouse strains (day -4) and prestimulated for 48 hours in Dulbecco's modified essential medium (DMEM; BioWhittaker, Walkersville, MD) supplemented with 15% fetal bovine serum, 100 units/ml penicillin, and 100 ng/ml streptomycin (P/S; Gibco-BRL). Growth factors were also included in the suspension culture at the following concentrations; 20 ng/ml murine IL-3 (Amgen), 50 ng/ml human IL-6 (Amgen), and 50 ng/ml murine SCF (Amgen and R & D Systems) as previously described. Following prestimulation (day -2), cells were co-cultured on irradiated (1500 rads) GP+E86 ecotropic producer cell lines for 48 hours in the presence of the same growth factor combination but also with added 6 mg/ml polybrene (Sigma) to enhance transduction. C57/Bl6 donor mice have a single hemoglobin (Hb) pattern while HW80 have a diffuse Hb pattern when separated on cellulose acetate gels (Helena Laboratories, Beaumont, Texas). These Hb patterns were subsequently utilized for characterization of engraftment.

Ex vivo culture and expansion of myeloid progenitors: Following transduction (day 0), cells were cultured in the presence of the growth factor combination described above. Cells were resuspended at 1 x 10^6 cells/ml every 3-6 days for at least 12 days

of expansion. Aliquots of cells were removed for CFU-C analysis at various time points. The percentage of drug-resistant progenitors was calculated by plating cells in methylcellulose (Stem Cell Technologies) in the presence of selective concentrations of drugs. MDR-transduced progenitors were resistant to 50 ng/ml taxol and DHFR-transduced progenitors were resistant to 25 to 50 nM trimetrexate. These concentrations of trimetrexate completely killed non-transduced background cells when plated in thymidine phosphorylase-treated methylcellulose. The percentage of transduced cells was found to remain constant through expansion. Average progenitor transduction efficiencies were: MDR-1 TaxolR (40.3 + 10.2%), DHFR TrimetrexateR (39.6 + 17.8%).

Non-irradiated recipient bone marrow transplants: During bone marrow transplant into non-irradiated recipients, mice received 5 daily intravenous injections with either MDR- or DHFR-transduced bone marrow cells (total of 20 - 40 x 10^6 cells for the 5 day period). Later each day mice also received intraperitoneal injection with trimetrexate (130 mg/kg) and the nucleoside transport inhibitor nitrobenzylmercaptopurine riboside phosphate (NBMPR-P; 20 mg/kg). Following this five day treatment course, the presence of donor Hb was monitored in recipient mice beginning at one week and followed for 8 to 14 months. Trimetrexate-glucuronate was received as the base from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI. It was converted to the glucuronate form as described previously [Spencer *et al.*, *Blood* **87**:2579-2587 (1996)].

Competitive repopulation assays: Expanded MDR-1 transduced cells were mixed either with the indicated donor hind limbs volume of 12 day expanded DHFRL22Y transduced cells or with freshly harvested marrow. Cells were mixed thoroughly and injected via the tail-vein into lethally-irradiated (925 - 1000 rads) recipient mice. Beginning at 10 weeks post-transplant, Hb patterns were analyzed by electrophoresis on cellulose acetate gels to calculate the relative proportions of single and diffuse

donor hemoglobin in reconstituted mice. The results of these analyses were quantitated by densitometry.

Secondary bone marrow transplants: Bone marrow was harvested from primary recipients from 10 to 24 weeks following transplant and injected into lethally-irradiated secondary recipients. Secondary transplanted mice received at least 5×10^6 bone marrow cells. Hb patterns were monitored in secondary recipients after reconstitution (8 to 10 weeks). Secondary CFU-S were harvested 12 days following injection of $1 - 5 \times 10^4$ cells and DNA was prepared for Southern blot analysis for the presence of the MDR-1 transgene.

Southern blot analysis: DNA was prepared as previously described [Sorrentino *et al.*, Science **257**: 99-103 (1992)]. Typically 10 to 20 mg of genomic DNA was restriction digested with either EcoR1 or NheI, and separated on a 1% agarose gel. Gels were blotted overnight onto Hybond N+ nylon membrane (Amersham), UV crosslinked, and hybridized with either MDR-1 or hemoglobin-specific [32P]-labeled probes. Blots were washed extensively at 65°C, exposed overnight, and analyzed on a phosphorimager (Molecular Dynamics).

Detection of human p-glycoprotein on mouse erythrocytes: One microliter of murine whole blood containing approximately 6×10^6 erythrocytes was washed in PBS and resuspended in 0.5 ml of Fc-Block (Pharmingen, San Diego, CA) in a final volume of 20 ml of PBS. The red cells were washed and resuspended in PBS with 0.3 mg of unlabeled primary anti-human Pgp monoclonal murine antibody 4E3 (M3523, Dako Corporation, Carpinteria, CA). Cells were next incubated with primary antibody for 45 minutes at room temperature. Cells were then washed and incubated with a phycoerythrin-linked goat anti-mouse IgG antibody (Caltag Laboratories, Burinlgame, CA) for 30 minutes at room temperature. Cells were next washed and analyzed by flow cytometry. Red cells and platelets were identified by the characteristic forward and side scatter distribution. Red cells were gated and analyzed for PE. Leukocytes

are located within the erythrocyte gate but these constitute less than 0.1% of all events.

Detection of human p-glycoprotein on mouse leukocytes: Murine whole blood was collected in heparinized tubes and diluted in PBS. Red blood cells were lysed in Gey's solution for 5 minutes on ice. White blood cells were blocked for 15 minutes in PBS/0.1% BSA/10% normal mouse serum followed by staining with a FITC-labeled murine monoclonal anti-human p-glycoprotein antibody (4E3-FITC; Signet Laboratories Inc., Dedham, MA) or with the isotype control. Cells were then analyzed by flow cytometry for FITC-positivity. Gates were drawn on the abnormal population apparent on forward and side scatter profiles in mice with a myeloproliferative disorder.

Assays for replication-competent retrovirus: Genomic DNA was prepared from either cultured producer cell lines, transduced 3T3 cells, or peripheral white blood cells. PCR was performed using primers specific for the 3' end of pol and the 5' end of env regions of the helper virus genome which have been previously described [Scarpa, *Virology* **180**:849 (1991)]. PCR was performed under the following conditions: 94°C, 1.5 minutes denaturation; 55°C, 1.0 minute annealing; 72°C, 1.5 minutes extension; 28 cycles. In addition, marker rescue assays were performed on supernatant from producer cell lines and plasma samples from transplanted mice. A M.dunni/G1Na-transduced cell line was used as the target for marker rescue. Following addition of supernatant, M. dunni/G1Na cells were cultured for at least 2 weeks and supernatant from these cells was assayed at intervals for liberation of infectious retrovirus containing the neo gene. Supernatant were transferred to naive M.dunni cells for 48 hours followed by selection in 0.8 mg/ml G418 (active). No G418R colonies were obtained in multiple experiments at a concentration in which M. dunni/G1Na cells were highly resistant.

Stem cell expansion calculation: 0.005/0.25 + 0.005 = 0.02 predicted engraftment percentage if no stem cell expansion had occurred. Observed engraftment levels =

0.25 to 1.0 indicate an increase in stem cell content of at least 10-fold. It should be noted that engraftment levels were constantly increasing thus any single measurement likely underestimates the true stem cell expansion.

Results

Ex vivo expansion of retrovirally-transduced murine myeloid progenitors was performed as follows. Bone marrow cells were harvested from either C57/Bl6 (C57) or B6.C-H1/BY (HW80) mice (day -4) and transduced by co-culture on retroviral producer cell lines. Producer cells included an MDR-1sc producer which expresses the splice-corrected version of the human MDR-1 cDNA and a resistance-conferring dihydrofolate reductase (DHFR) mutant referred to as DHFRL22Y. Following transduction (day 0), cells placed into liquid suspension culture for a period of 12 days expanded logarithmically (Fig. 1A). At three day intervals, an aliquot of cells was removed and plated in methylcellulose for assay of the progenitor content. Total progenitor and drug-resistant progenitor levels were calculated and monitored over time. The percentage of progenitors within the total cell population peaked at levels close to 20% between 3 and 6 days following initiation of culture and then gradually declined with time. This transient relative enrichment in progenitors is likely due to death of differentiated cells and expansion of the progenitor pool. The absolute numbers of both drug-resistant and drug-sensitive progenitor populations expanded equally during culture. Thus, the relative percentage of drug-resistant progenitors remained constant throughout the 12 days in culture. Examples of representive expansions are shown for both MDR-1 and DHFR-transduced drug-resistant progenitors (Fig. 1B). Typical expansions yielded a 100-fold increase by 2 weeks.

Long-term engraftment of MDR-transduced hematopoietic stem cells was observed in non-irradiated recipient mice. The expansion of cells capable of long-term engraftment in a non-irradiated mouse model was unexpected. MDR- or DHFR-transduced and expanded cells were initially injected into mice with the purpose of testing whether short-term engraftment of drug-resistant progenitors would be

protective against antifolate-induced myelosuppression. Recipient mice were treated for 5 days with trimetrexate in combination with the nucleoside transport inhibitor nitrobenzylmercaptopurine riboside phosphate (NBMPR-P). Mice were injected with 12-16 day-expanded cells on each of the 5 days of drug treatment. Following transplant the donor hemoglobin (Hb) levels were monitored in the recipient mice beginning at 1 week and continued through greater than 1 year following injection (Fig. 2A-2B). Donor Hb was present in varying levels in all mice receiving cells as early as 1 week following injection (the earliest time point examined). However, this engraftment was only transient in mice receiving DHFR- or mock-transduced marrow (0/16; from 2 separate expansion experiments, Fig.2A). By contrast, 5/12 mice which received MDR-transduced marrow showed long-term engraftment which was stable for more than 6 months post BMT (Fig. 2B), and up to 14 months in the latest time point obtained. Representative Hb electrophoresis profiles for primary engrafted recipients demonstrated the presence of C57 donor Hb at time points 5 to 7 months following injection (Fig. 3A-3B). In addition, secondary recipients from mouse #20 showed a range from 50 - 100% C57 donor Hb 8 weeks after transplant indicating engraftment of the primitive long-term repopulating cells (Fig. 3C). High level expression of P-glycoprotein was seen in donor red blood cells at greater than 10 weeks following transplant in all 4 mice engrafted from experiment #1 (Fig. 3D). The FACS data shows expression in total red blood cells and when corrected for donor chimerism at the same day of analysis the levels were found to range from 80 - 100% positivity. DHFR mouse #1 served as a negative control for P-glycoprotein expression.

Expansion of the MDR-transduced hematopoietic stem cells was quantified by competitive repopulation assay. Thus, to examine whether stem cell expansion was responsible for the high levels of engraftment obtained in the non-irradiated model, a competitive repopulation model was used. MDR- and DHFR-transduced cells were expanded over a 12 day period as described in the Methods, above. Table 1 shows the calculation of the percent hind limbs volume remaining at various time points during the expansion. On day 12, MDR-transduced cells were mixed in an equal

volume:volume ratio with expanded day 12 DHFR-transduced cells and injected into lethally-irradiated recipient mice (Figure 4A, right). Also, expanded MDR cells (0.005 C57 donor volumes, Figure 4A, left) or expanded DHFR cells (0.005 C57 donor volumes Figure 4A, middle) were mixed with freshly isolated cells with the opposing Hb pattern (0.25 donor volumes) and injected into lethally-irradiated recipients. Beginning at 10 weeks the Hb patterns in recipient mice were analyzed by Hb electrophoresis (Figure 4C). Interestingly, MDR-expanded marrow completely out competed identically expanded DHFR marrow in repopulation of recipient mice indicating a much higher stem cell content, (lanes 4 -10 of Figure 4C). But most surprising was the very high level of engraftment of MDR marrow when competed against fresh marrow (lanes 1 and 2 of Figure 4C.). By contrast, when DHFR marrow was competed against fresh marrow it was completely outcompeted (lanes 3 and 4 of Fig. 4C). In addition, over time mice consistently lost their chimerism and approached 100% donor as was seen in the unirradiated model.

TABLE 1

Calculation of Total Cell Expansion and

Percent Hind Limbs Volume Remaining

	HaMDR			HaDHFR		
Day	**Cell Number***	**Volume fraction replated**	**Fraction hind limbs volume**	**Cell Number***	**Volume fraction replated**	**Fraction hind limbs volume**
0	2.15×10^7	1.00	0.86	2.06×10^7	1.00	1.00
3	9.2×10^7	0.22	0.19	6.2×10^7	0.22	0.22
6	6.6×10^7	0.40	0.076	6×10^7	0.52	0.114
9	8×10^7	1.00	0.076	7.9×10^7	1.00	0.114
12	1.6×10^7	---	0.076	8.2×10^7	---	0.114

Note: On days 3 and 6 a fraction of the cells were replated while the rest were discarded. This is reflected in the fraction hind limbs volume remaining. After 12 days of expansion, 0.005 hind limb volumes were injected per mouse along with 0.25 for fresh competed marrow. The fraction at day 0 was determined by the percent of the total volume used following flushing the bone marrow from both fibias and femurs of a single mouse.

* Cell number values are those prior to cell replating

Expanded stem cells are marked with MDR-1 proviral DNA. To determine whether the engrafted donor cells were transduced with the MDR-1 virus, secondary day 12 CFU-S were analyzed by southern blot (Fig. 5). Genomic DNA from individual CFU-S were digested with EcoR1 and probed with an MDR-1 specific probe. A total of 88/88 CFU-S from 7 primary recipients (6 MDR vs. DHFR mice from competitive repopulation expt. #1, and MDR #15 from non-irradiated expt. #2) were shown to be positive by southern blot for the MDR-1 provirus giving a band of the expected size (3464 bp). In addition, a subset of samples were digested with NheI which indicated the presence of the correct size full-length retroviral transcript (8580 bp). These data link the presence of the transgene and expansion of primitive stem cells.

The correlation of the stem cell expansion with period of time in culture was next investigated. To determine the kinetics of stem cell expansion during culture, an additional competitive repopulation experiment was performed. For this experiment, the donor and recipient hemoglobins were switched to eliminate any possibility that engraftment was related to the donor Hb pattern. Following transduction with the MDR-1 retrovirus, cells were cultured and aliquots competed at a ratio of 0.02 vol. MDR (HW80)/0.25 vol. fresh (C57) on days 0, 3, 6, and 12. Engraftment of MDR-1 marrow was only seen following at least 3 days of expansion post co-culture and engraftment increased with increasing time in culture (Figure 6A). In order to determine whether the high levels of engraftment shown in the erythroid lineage by hemoglobin electrophoresis were also maintained in other lineages, Southern blot analysis was performed on peripheral blood DNA. Blots were treated with the restriction enzyme EcoR1 and probed with a hemoglobin allele-specific probe (Figure 6B). The results shown in Figure 6B demonstrate that the levels of donor hemoglobin seen in the peripheral blood and the levels of lympho-myeloid reconstitution represented in the peripheral blood cell DNA are in full agreement.

In some of the transplanted mice a myeloproliferative disorder was observed. However, the myeloproliferative disorder is not a necessary consequence of the engraftment of the expanded gene-modified hematopoietic stem cells transduced with a nucleic acid encoding MDR-1. Engrafted mice were analyzed serially to monitor the level of engraftment over time. In some mice, it was noticed that the peripheral white blood cell (WBC) counts began to rise to abnormal levels (Figure 7A). Shown in Figure 7A are 10 representative engrafted mice from the competitive repopulation experiment #2, above. 2/10 of these mice retain normal WBC counts at the present time despite a very large stem cell expansion. In most of the cases the elevation was extremely rapid and could increase by as much as 10-fold within a few days. White blood cell counts at last analysis ranged from 97,018 to as high as 388,433 cells/ml for MDR mice compared with normal values of 8654 + 2626 ($p<0.001$). Analysis of Wright-stained blood smears (Fig. 7B) showed a relative increase in an abnormal cell population (bottom 2 panels relative to normal mouse in the top panel).

Immunophenotyping of the population revealed a high percentage of GR-1 and MAC-1 positive cells, consistent with the granulocytic morphology of the majority of these cells. In a few instances, blasts were seen in the peripheral blood consistent with progression into leukemia. These cells did not stain positively for any lineage marker. The disease was found to be transplantable into secondary recipients which rapidly developed the same increases in white cell counts.

Replication-competent retrovirus (RCR) assays were also performed extensively on both cell lines and plasma from mice with the myeloproliferative disorder. A very sensitive PCR assay for helper virus failed to detect the helper genome but was highly positive when using positive control monkey DNA. In addition, marker rescue assays on Mus dunni cells eliminated the possibility of contamination with retroviruses of a wide host range. These data indicate that the stem cell expansion and subsequent myeloproliferative disorder are not due to a contamination of helper virus.

In addition to the elevated WBC count, the number of clonogenic progenitors in the peripheral blood and spleen increased dramatically. Typical progenitor numbers in the blood of a normal animal were 1 - 4/105 cells. Progenitor counts in some mice ranged from 57 to 1290/105 cells. Splenomegaly was also seen in mice with the myeloproliferative disorder. Spleen weights ranged from 483 to 834 mg compared to 106 + 48 mg for normal mice. The progenitor content in the spleen was concomitantly increased from a normal of 3.3-18/105 to 180/105 cells.

Importantly, despite the abnormal hematologic phenotype, the mice appeared grossly normal and healthy even with the highest white blood cell counts. Analysis of the bone marrow revealed no morphological abnormalities consistent with leukemia. In addition, the mouse karyotype was normal and there were no chromosome translocations present in peripheral blood metaphases from the two representative mice examined. These data are consistent with a prolonged period of abnormal myeloproliferation with transformation to leukemia in only a minority of mice. Peripheral blood cells from several diseased mice were also injected into SCID mice

without the development of tumors. Importantly, a percentage of mice have shown large increases in stem cell content and have maintained normal hematologic parameters for as long as 9 to 14 months following transplant. The myeloproliferative syndrome can be dissociated from the hematopoietic stem cell expansion as shown by a significant number of healthy transplanted mice in which there was no evidence of myeloproliferative syndrome.

The following is a list of documents related to the above disclosure and particularly to the experimental procedures and discussions. These documents, and all others cited above, should be considered as incorporated by reference in their entirety.

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While the invention has been described and illustrated herein by references to the specific embodiments, various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications in addition to the immediately foregoing are cited herein, the disclosures of which are also incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method of performing *ex vivo* expansion of a gene-modified hematopoietic stem cell comprising:

(a) transducing a hematopoietic stem cell with a nucleic acid encoding MDR-1; wherein the hematopoietic stem cell is transduced to become a gene-modified hematopoietic stem cell; and

(b) culturing the gene-modified hematopoietic stem cell *ex vivo* wherein the gene-modified hematopoietic stem cell is expanded.

2. The method of Claim 1 wherein said culturing is performed in the presence of an early-acting hematopoietic cytokine.

3. The method of Claim 1 wherein the cytokine is selected from the group of cytokines consisting of interleukin-3, interleukin-6, G-CSF, GM-CSF, FLT-3 ligand, and stem cell factor.

4. The method of Claim 1 wherein transducing the hematopoietic stem cell with a nucleic acid encoding MDR-1 is performed with a viral vector comprising a nucleic acid encoding an MDR-1.

5. The method of Claim 4 wherein the viral vector is selected from the group of viral vectors consisting of a herpes simplex viral vector, an adenoviral vector, and adeno-associated viral vector (AAV).

6. The method of Claim 4 wherein the viral vector is a retroviral vector.

7. The method of Claim 6 wherein the retroviral vector is a Harvey Murine Sarcoma Vector and the hematopoietic stem cell is transduced by co-culture on retroviral producer cell lines.

8. The method of Claim 1 wherein transducing the hematopoietic stem cell with a nucleic acid encoding MDR-1 is performed with a DNA vector comprising a nucleic acid encoding an MDR-1.

9. The method of Claim 1 wherein the hematopoietic stem cell is a mammalian hematopoietic stem cell.

10. The method of Claim 9 wherein the gene-modified hematopoietic stem cell expresses a splice-corrected version of the human MDR-1.

11. The method of Claim 9 wherein the mammalian hematopoietic stem cell is a murine hematopoietic stem cell.

12. The method of Claim 9 wherein the mammalian hematopoietic stem cell is a human hematopoietic stem cell.

13. A gene-modified mammalian hematopoietic stem cell that has been (i) transduced with a nucleic acid encoding MDR-1, and (ii) expanded.

14. The gene-modified hematopoietic stem cell of Claim 13 that has been expanded for at least 9 days.

15. A method of engrafting a mammal with the gene-modified hematopoietic stem cell of Claim 13 comprising placing said cell into the mammal.

16. The method of Claim 15 wherein the gene-modified hematopoietic stem cell further comprises a heterologous gene, and wherein the expression of the heterologous gene by the gene-modified hematopoietic stem cell in the mammal aids in the treatment of a disease associated with a dysfunctional cell; wherein said dysfunctional cell is derived from an hematopoietic stem cell.

17. The method of Claim 15 wherein said placing is performed by injecting.

18. The method of Claim 17 wherein said injecting is repeated more than once.

19. The method of Claim 15 wherein said engrafting is stable for at least six months.

20. The method of Claim 15 wherein said mammal is a human.

FIG. 1A

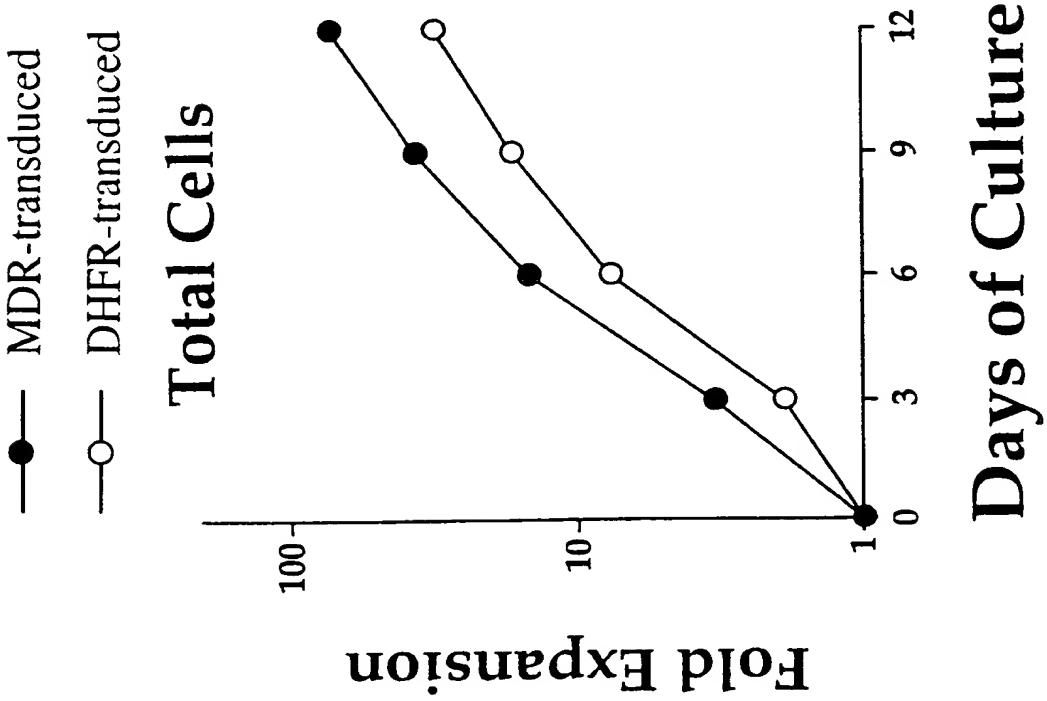
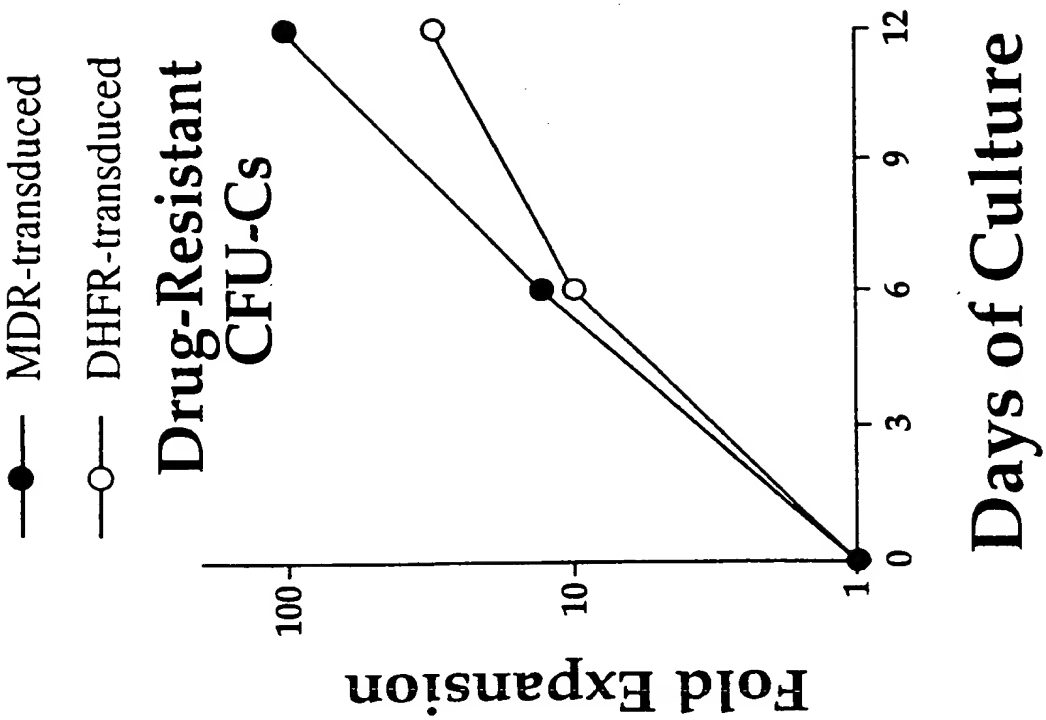

MDR-transduced
DHFR-transduced
Total Cells
Fold Expansion
100
10
1
0
3
6
9
12
Days of Culture

FIG. 1B


MDR-transduced
DHFR-transduced
Drug-Resistant CFU-Cs
Fold Expansion
100
10
1
0
3
6
9
12
Days of Culture

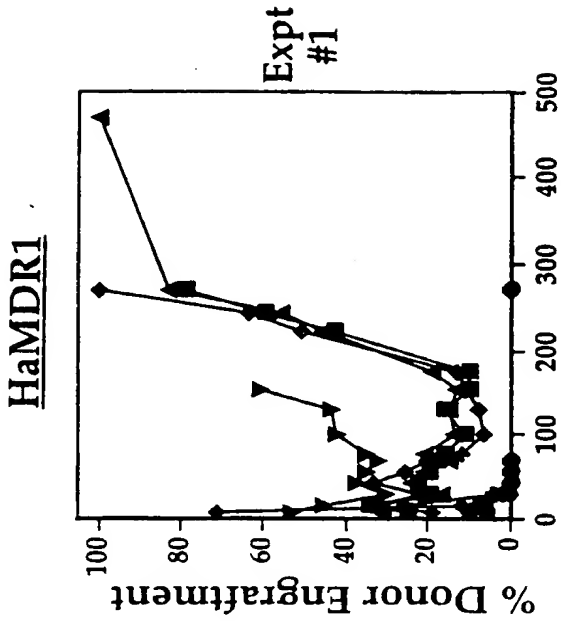


FIG. 2C

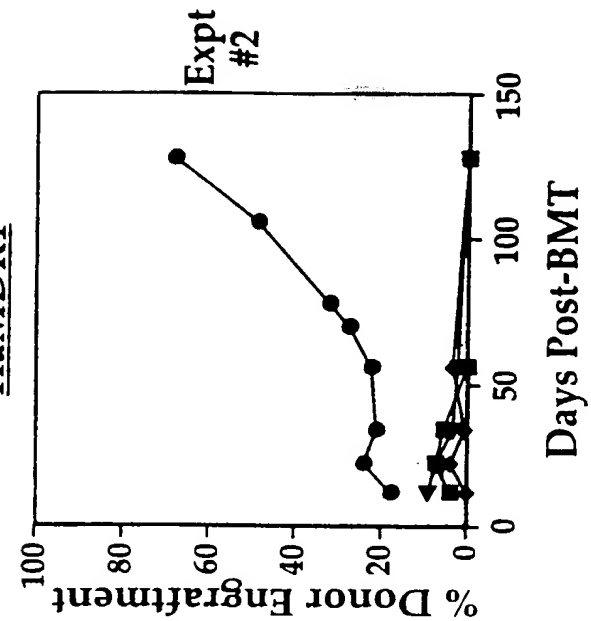


FIG. 2D

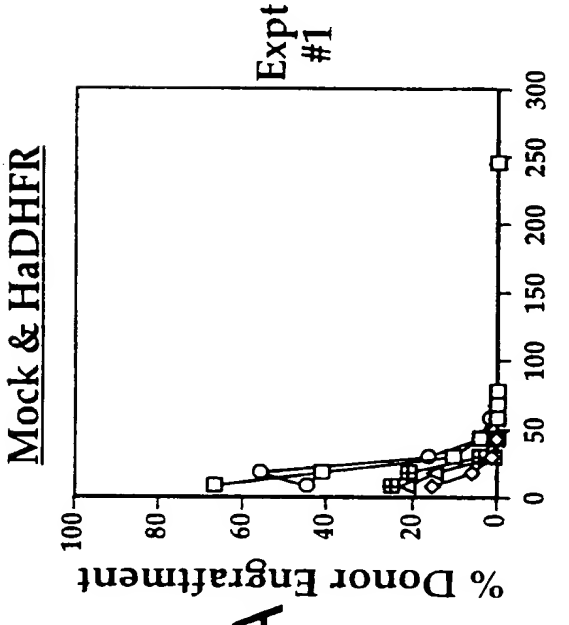


FIG. 2A

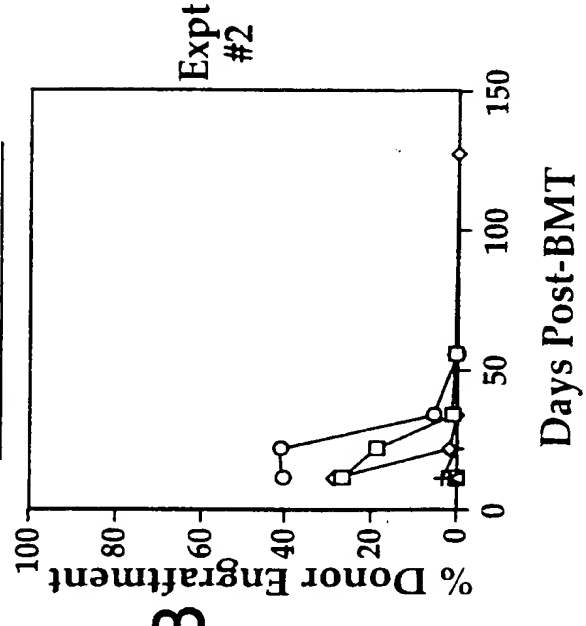


FIG. 2B

FIG. 3A

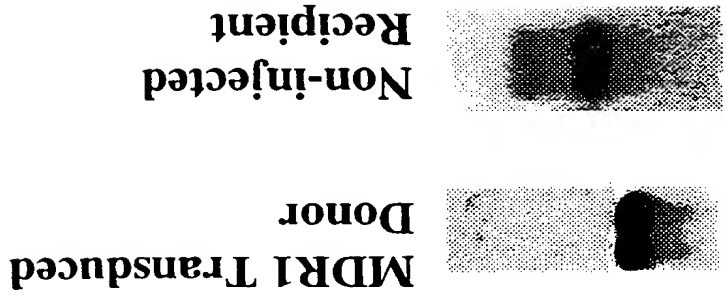


FIG. 3B

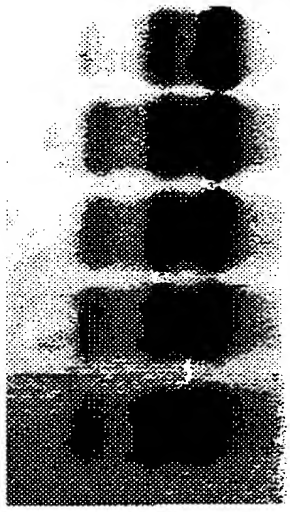


FIG. 3C

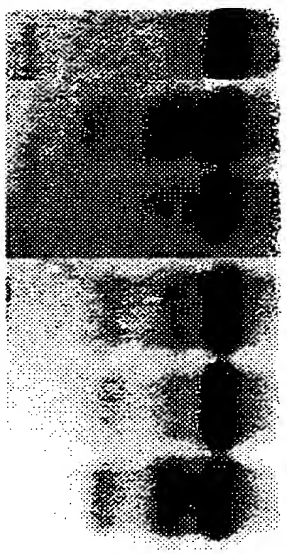


FIG. 3D-1

EVENTS

FIG. 3D-2

EVENTS

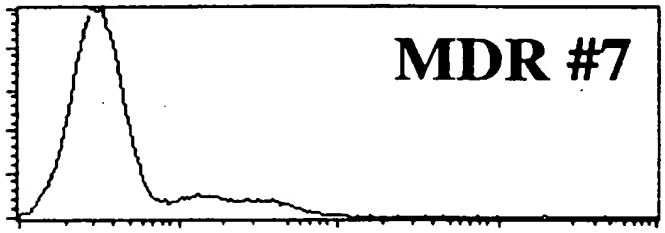


FIG. 3D-3

EVENTS

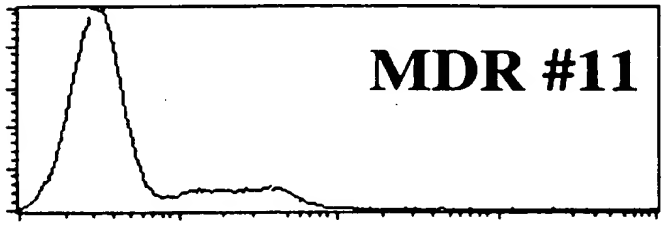


FIG. 3D-4

EVENTS

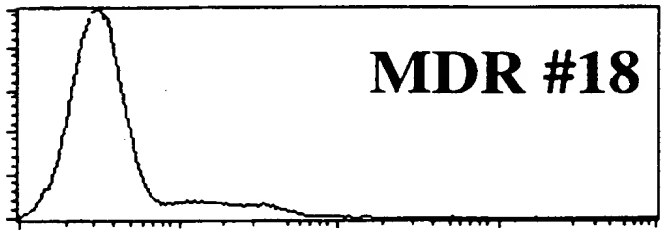
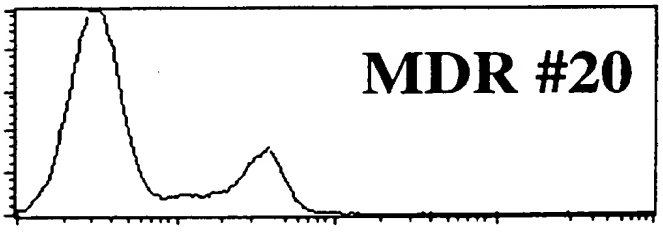


FIG. 3D-5

EVENTS



PgP Fluoresence

FIG. 4

Donor cells	C57 vs. HW80	HW80 vs. C57	C57 vs. HW80
Vector:Days Expansion	MDR:12 vs. None:0	DHFR:12 vs.None:0	MDR:12 vs. DHFR:12
Fraction Hind Limbs Vol.	0.005 vs. 0.25	0.005 vs.0.25	0.005 vs. 0.005

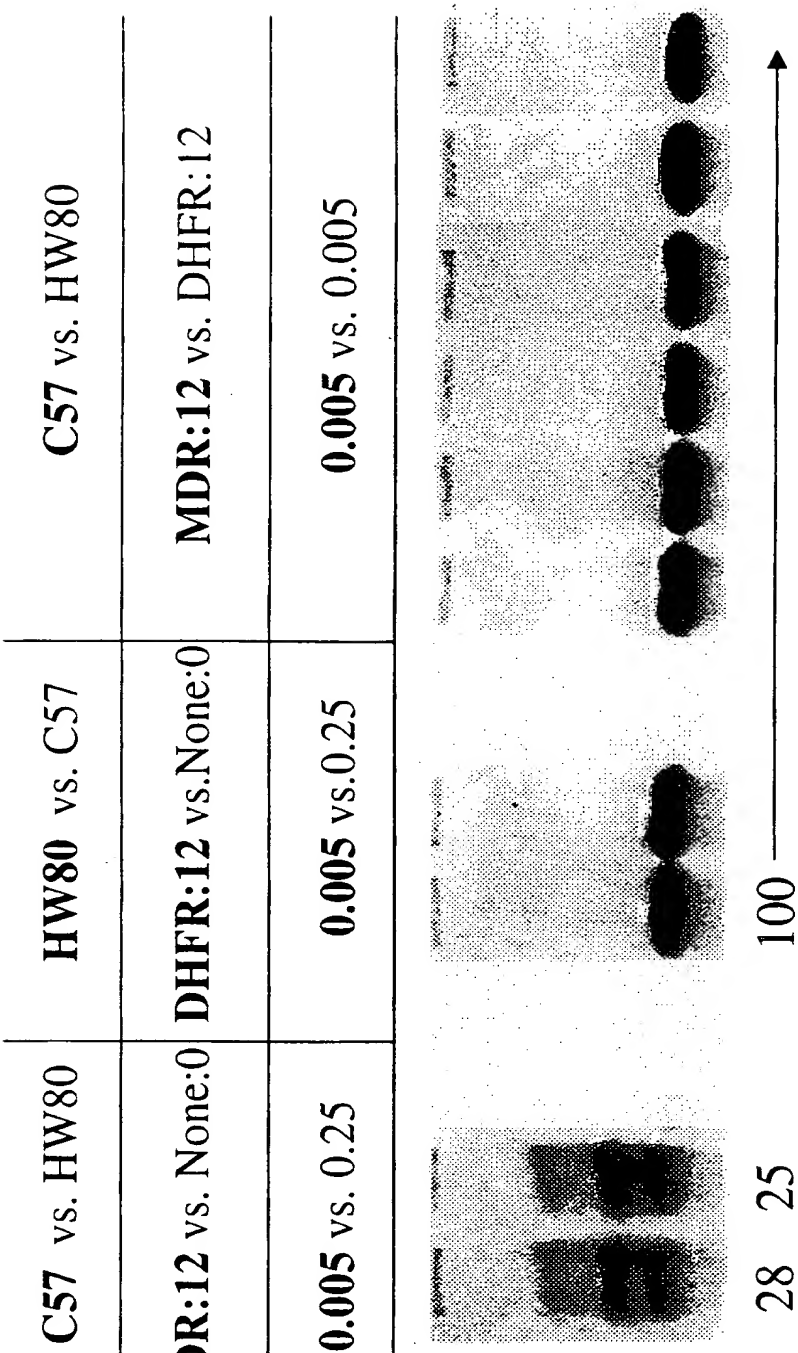


FIG. 5

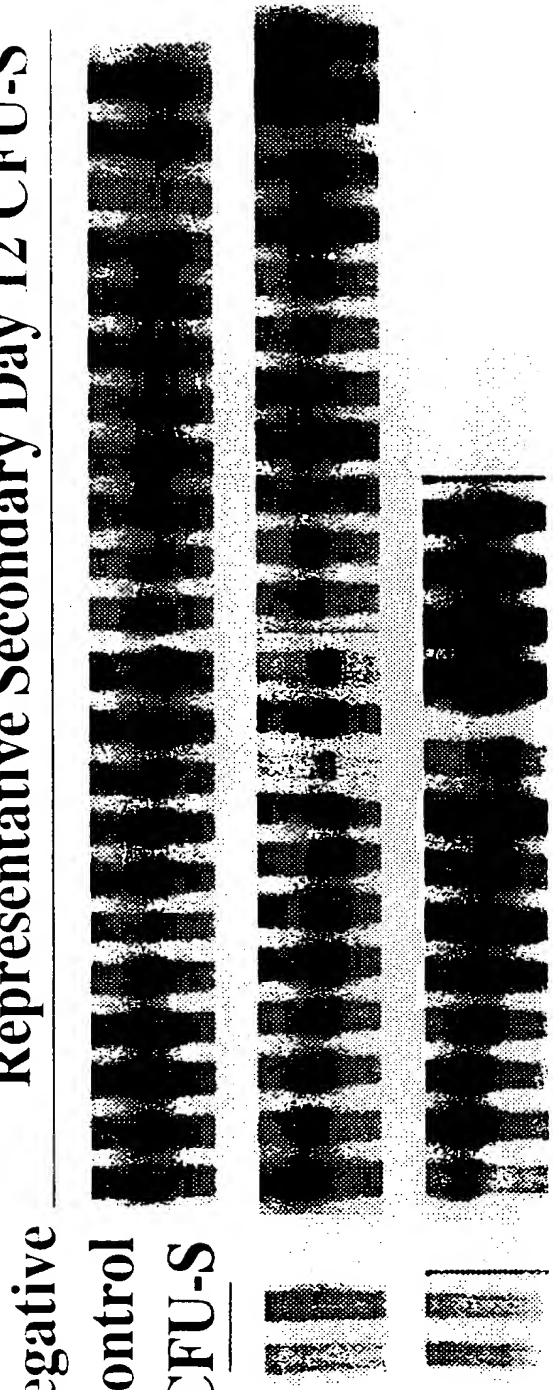

Representative Secondary Day 12 CFU-S
Negative Control
CFU-S

FIG. 6A

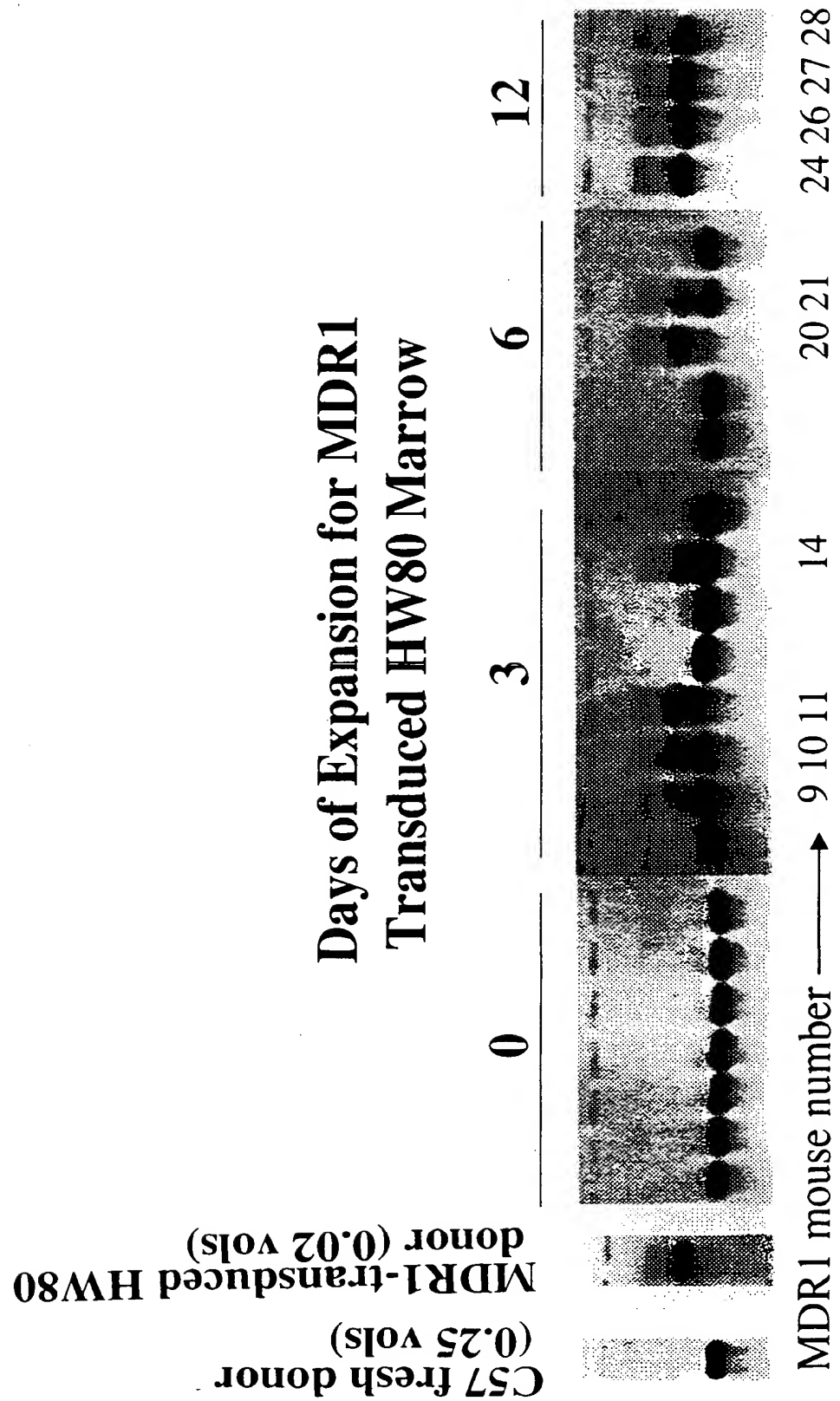
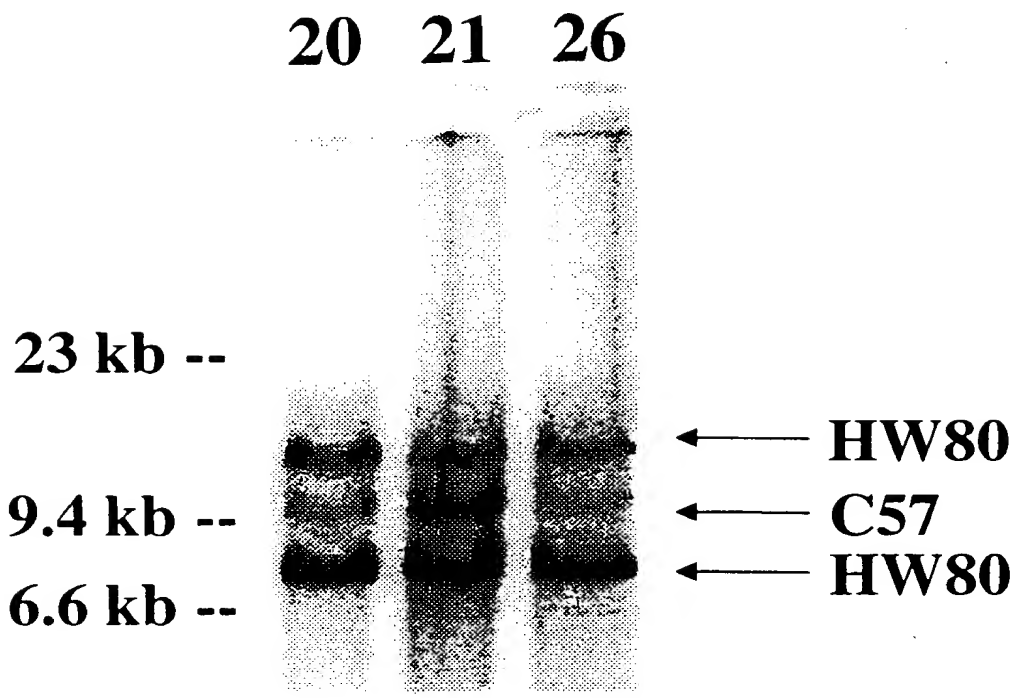
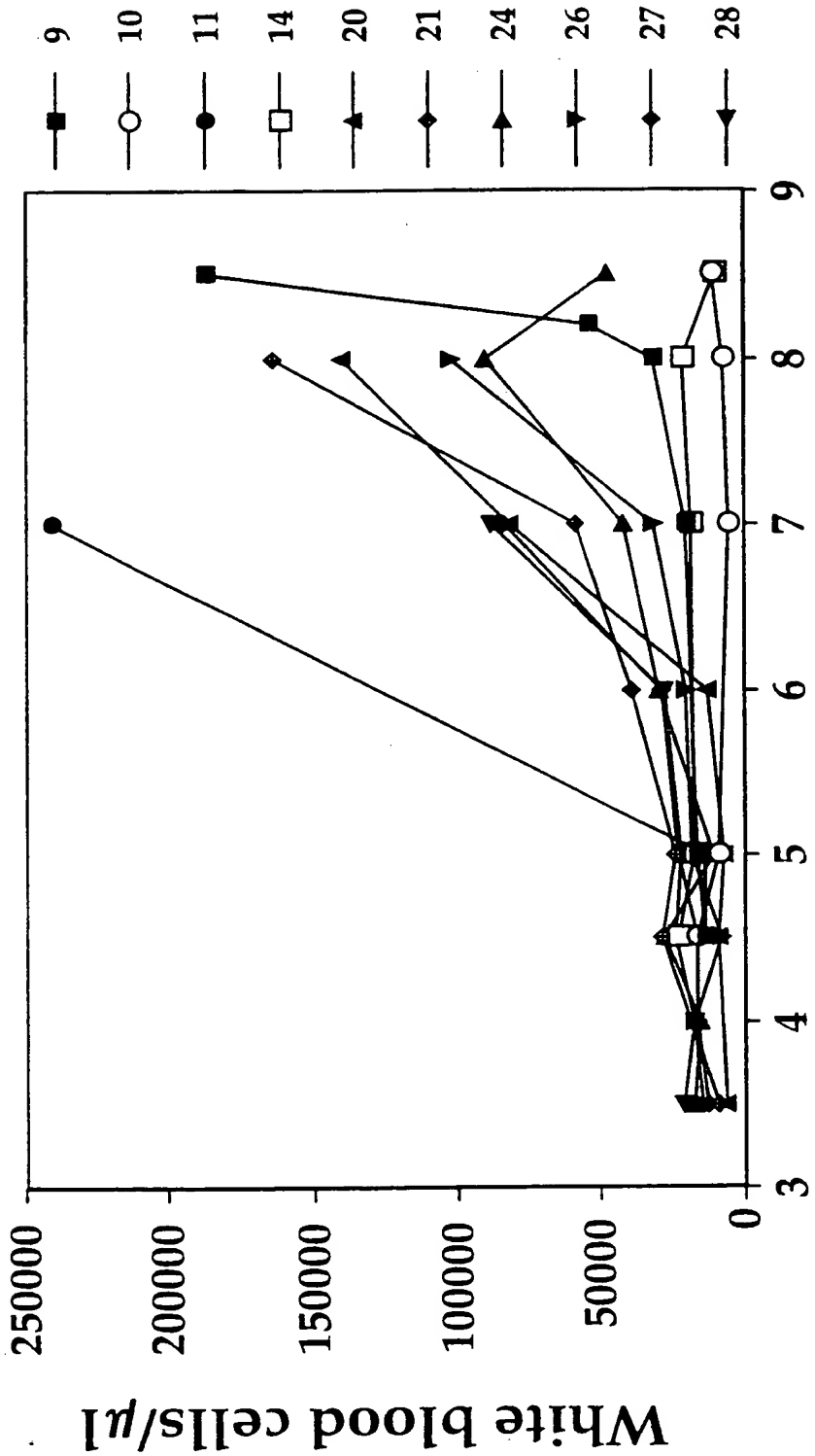

Days of Expansion for MDR1 Transduced HW80 Marrow
0
3
6
12
C57 fresh donor (0.25 vols)
MDR1-transduced HW80 donor (0.02 vols)
MDR1 mouse number
9 10 11
14
20 21
24 26 27 28

FIG. 6B




9
10
11
14
20
21
24
26
27
28
White blood cells/μl
250000
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150000
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50000
0
3
4
5
6
7
8
9
Months Post-Transplant

FIG. 7A

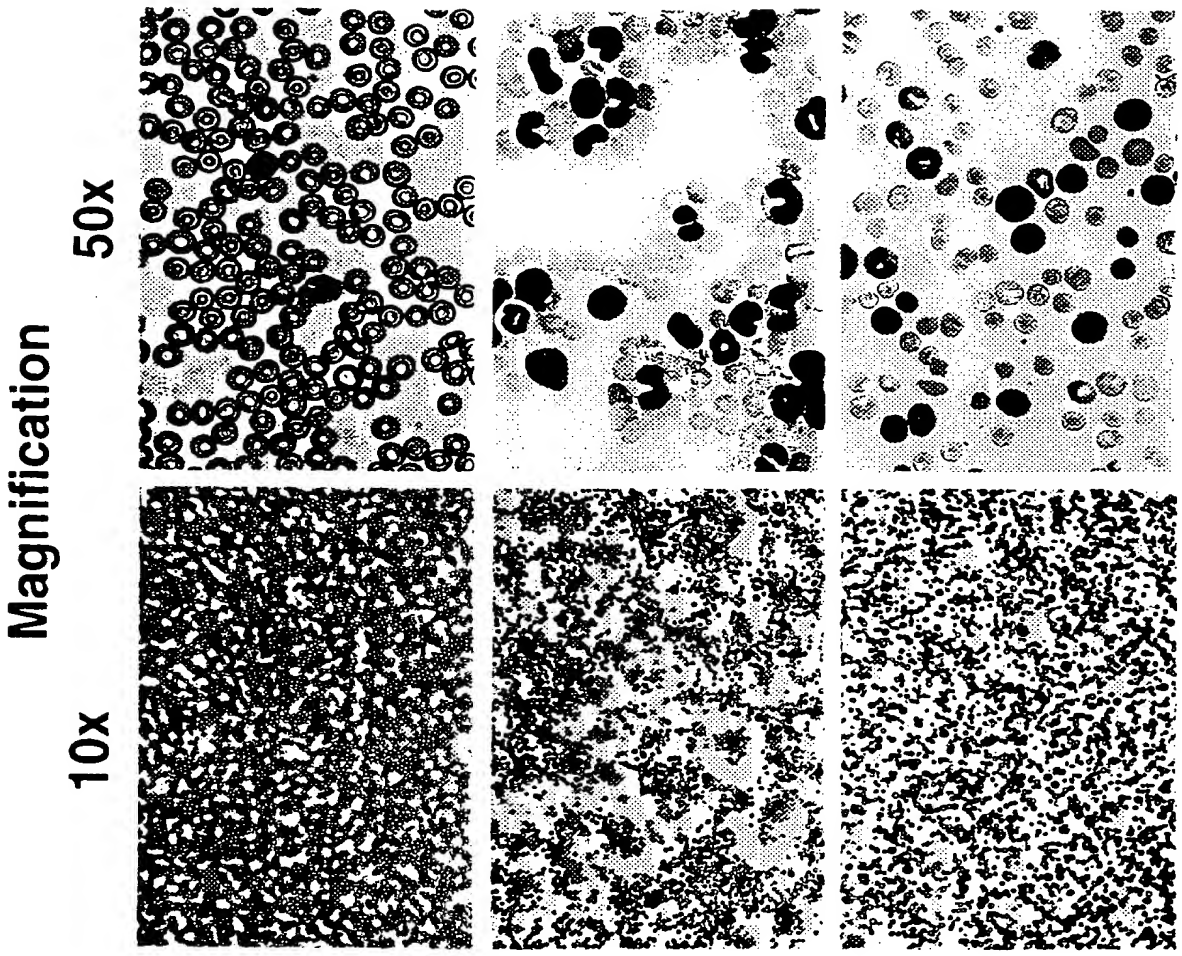

FIG. 7B-1 Normal

FIG. 7B-2 Normal

FIG. 7B-3 Abnormal

FIG. 7B-4 Abnormal

FIG. 7B-5 Abnormal

FIG. 7B-6 Abnormal

SEQUENCE LISTING

<110> SORRENTINO ET AL.

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Leu Pro Leu Met Met Leu Val Phe Gly Glu Met Thr Asp Ile Phe Ala
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His Lys Ile Arg Lys Gln Phe Phe His Ala Ile Met Arg Gln Glu Ile
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Asp Asp Val Ser Lys Ile Asn Glu Gly Ile Gly Asp Lys Ile Gly Met
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the pamphlet!

WO 99-61589

2/2

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gcaaatcttg ggacaggaat aattatatcc ttcatctatg gttggcaact aacactgtta 2580

ctcttagcaa ttgtacccat cattgcaata gcaggagttg ttgaaatgaa aatgttgtct 2640

ggacaagcac tgaaagataa gaaagaacta gaaggtgctg ggaagatcgc tactgaagca 2700

atagaaaact tccgaaccgt tgtttctttg actcaggagc agaagtttga acatatgtat 2760

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gacttaatag tggtgtttca gaatggcaga gtcaaggagc atggcacgca tcagcagctg 3780

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<211> 1280

<212> PRT

<213> HUMAN MDR 185-V

<400> 4

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Phe Lys Leu Asn Asn Lys Ser Glu Lys Asp Lys Lys Glu Lys Lys Pro
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Thr Val Ser Val Phe Ser Met Phe Arg Tyr Ser Asn Trp Leu Asp Lys
35 40 45

Leu Tyr Met Val Val Gly Thr Leu Ala Ala Ile Ile His Gly Ala Gly
50 55 60

Leu Pro Leu Met Met Leu Val Phe Gly Glu Met Thr Asp Ile Phe Ala
65 70 75 80

Asn Ala Gly Asn Leu Glu Asp Leu Met Ser Asn Ile Thr Asn Arg Ser
85 90 95

Asp Ile Asn Asp Thr Gly Phe Phe Met Asn Leu Glu Glu Asp Met Thr

100 105 110

Arg Tyr Ala Tyr Tyr Tyr Ser Gly Ile Gly Ala Gly Val Leu Val Ala
115 120 125

Ala Tyr Ile Gln Val Ser Phe Trp Cys Leu Ala Ala Gly Arg Gln Ile
130 135 140

His Lys Ile Arg Lys Gln Phe Phe His Ala Ile Met Arg Gln Glu Ile
145 150 155 160

Gly Trp Phe Asp Val His Asp Val Gly Glu Leu Asn Thr Arg Leu Thr
165 170 175

Asp Asp Val Ser Lys Ile Asn Glu Val Ile Gly Asp Lys Ile Gly Met
180 185 190

Phe Phe Gln Ser Met Ala Thr Phe Phe Thr Gly Phe Ile Val Gly Phe
195 200 205

Thr Arg Gly Trp Lys Leu Thr Leu Val Ile Leu Ala Ile Ser Pro Val
210 215 220

Leu Gly Leu Ser Ala Ala Val Trp Ala Lys Ile Leu Ser Ser Phe Thr
225 230 235 240

Asp Lys Glu Leu Leu Ala Tyr Ala Lys Ala Gly Ala Val Ala Glu Glu
245 250 255

Val Leu Ala Ala Ile Arg Thr Val Ile Ala Phe Gly Gly Gln Lys Lys
260 265 270

Glu Leu Glu Arg Tyr Asn Lys Asn Leu Glu Glu Ala Lys Arg Ile Gly
275 280 285

Ile Lys Lys Ala Ile Thr Ala Asn Ile Ser Ile Gly Ala Ala Phe Leu
290 295 300

Leu Ile Tyr Ala Ser Tyr Ala Leu Ala Phe Trp Tyr Gly Thr Thr Leu
305 310 315 320

Val Leu Ser Gly Glu Tyr Ser Ile Gly Gln Val Leu Thr Val Phe Phe
325 330 335

Ser Val Leu Ile Gly Ala Phe Ser Val Gly Gln Ala Ser Pro Ser Ile
340 345 350

Glu Ala Phe Ala Asn Ala Arg Gly Ala Ala Tyr Glu Ile Phe Lys Ile
355 360 365

Ile Asp Asn Lys Pro Ser Ile Asp Ser Tyr Ser Lys Ser Gly His Lys
370 375 380

Pro Asp Asn Ile Lys Gly Asn Leu Glu Phe Arg Asn Val His Phe Ser
385 390 395 400

Tyr Pro Ser Arg Lys Glu Val Lys Ile Leu Lys Gly Leu Asn Leu Lys
405 410 415

Val Gln Ser Gly Gln Thr Val Ala Leu Val Gly Asn Ser Gly Cys Gly
420 425 430

Lys Ser Thr Thr Val Gln Leu Met Gln Arg Leu Tyr Asp Pro Thr Glu
435 440 445

Gly Met Val Ser Val Asp Gly Gln Asp Ile Arg Thr Ile Asn Val Arg
450 455 460

Phe Leu Arg Glu Ile Ile Gly Val Val Ser Gln Glu Pro Val Leu Phe
465 470 475 480

Ala Thr Thr Ile Ala Glu Asn Ile Arg Tyr Gly Arg Glu Asn Val Thr
485 490 495

Met Asp Glu Ile Glu Lys Ala Val Lys Glu Ala Asn Ala Tyr Asp Phe
500 505 510

Ile Met Lys Leu Pro His Lys Phe Asp Thr Leu Val Gly Glu Arg Gly
515 520 525

Ala Gln Leu Ser Gly Gly Gln Lys Gln Arg Ile Ala Ile Ala Arg Ala
530 535 540

Leu Val Arg Asn Pro Lys Ile Leu Leu Leu Asp Glu Ala Thr Ser Ala
545 550 555 560

Leu Asp Thr Glu Ser Glu Ala Val Val Gln Val Ala Leu Asp Lys Ala

565 570 575

Arg Lys Gly Arg Thr Thr Ile Val Ile Ala His Arg Leu Ser Thr Val
580 585 590

Arg Asn Ala Asp Val Ile Ala Gly Phe Asp Asp Gly Val Ile Val Glu
595 600 605

Lys Gly Asn His Asp Glu Leu Met Lys Glu Lys Gly Ile Tyr Phe Lys
610 615 620

Leu Val Thr Met Gln Thr Ala Gly Asn Glu Val Glu Leu Glu Asn Ala
625 630 635 640

Ala Asp Glu Ser Lys Ser Glu Ile Asp Ala Leu Glu Met Ser Ser Asn
645 650 655

Asp Ser Arg Ser Ser Leu Ile Arg Lys Arg Ser Thr Arg Arg Ser Val
660 665 670

Arg Gly Ser Gln Ala Gln Asp Arg Lys Leu Ser Thr Lys Glu Ala Leu
675 680 685

Asp Glu Ser Ile Pro Pro Val Ser Phe Trp Arg Ile Met Lys Leu Asn
690 695 700

Leu Thr Glu Trp Pro Tyr Phe Val Val Gly Val Phe Cys Ala Ile Ile
705 710 715 720

Asn Gly Gly Leu Gln Pro Ala Phe Ala Ile Ile Phe Ser Lys Ile Ile
725 730 735

Gly Val Phe Thr Arg Ile Asp Asp Pro Glu Thr Lys Arg Gln Asn Ser
740 745 750

Asn Leu Phe Ser Leu Leu Phe Leu Ala Leu Gly Ile Ile Ser Phe Ile
755 760 765

Thr Phe Phe Leu Gln Gly Phe Thr Phe Gly Lys Ala Gly Glu Ile Leu
770 775 780

Thr Lys Arg Leu Arg Tyr Met Val Phe Arg Ser Met Leu Arg Gln Asp
785 790 795 800

Val Ser Trp Phe Asp Asp Pro Lys Asn Thr Thr Gly Ala Leu Thr Thr
805 810 815

Arg Leu Ala Asn Asp Ala Ala Gln Val Lys Gly Ala Ile Gly Ser Arg
820 825 830

Leu Ala Val Ile Thr Gln Asn Ile Ala Asn Leu Gly Thr Gly Ile Ile
835 840 845

Ile Ser Phe Ile Tyr Gly Trp Gln Leu Thr Leu Leu Leu Leu Ala Ile
850 855 860

Val Pro Ile Ile Ala Ile Ala Gly Val Val Glu Met Lys Met Leu Ser
865 870 875 880

Gly Gln Ala Leu Lys Asp Lys Lys Glu Leu Glu Gly Ala Gly Lys Ile
885 890 895

Ala Thr Glu Ala Ile Glu Asn Phe Arg Thr Val Val Ser Leu Thr Gln
900 905 910

Glu Gln Lys Phe Glu His Met Tyr Ala Gln Ser Leu Gln Val Pro Tyr
915 920 925

Arg Asn Ser Leu Arg Lys Ala His Ile Phe Gly Ile Thr Phe Ser Phe
930 935 940

Thr Gln Ala Met Met Tyr Phe Ser Tyr Ala Gly Cys Phe Arg Phe Gly
945 950 955 960

Ala Tyr Leu Val Ala His Lys Leu Met Ser Phe Glu Asp Val Leu Leu
965 970 975

Val Phe Ser Ala Val Val Phe Gly Ala Met Ala Val Gly Gln Val Ser
980 985 990

Ser Phe Ala Pro Asp Tyr Ala Lys Ala Lys Ile Ser Ala Ala His Ile
995 1000 1005

Ile Met Ile Ile Glu Lys Thr Pro Leu Ile Asp Ser Tyr Ser Thr Glu
1010 1015 1020

Gly Leu Met Pro Asn Thr Leu Glu Gly Asn Val Thr Phe Gly Glu Val

1025 1030 1035 1040

Val Phe Asn Tyr Pro Thr Arg Pro Asp Ile Pro Val Leu Gln Gly Leu
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Ser Leu Glu Val Lys Lys Gly Gln Thr Leu Ala Leu Val Gly Ser Ser
1060 1065 1070

Gly Cys Gly Lys Ser Thr Val Val Gln Leu Leu Glu Arg Phe Tyr Asp
1075 1080 1085

Pro Leu Ala Gly Lys Val Leu Leu Asp Gly Lys Glu Ile Lys Arg Leu
1090 1095 1100

Asn Val Gln Trp Leu Arg Ala His Leu Gly Ile Val Ser Gln Glu Pro
1105 1110 1115 1120

Ile Leu Phe Asp Cys Ser Ile Ala Glu Asn Ile Ala Tyr Gly Asp Asn
1125 1130 1135

Ser Arg Val Val Ser Gln Glu Glu Ile Val Arg Ala Ala Lys Glu Ala
1140 1145 1150

Asn Ile His Ala Phe Ile Glu Ser Leu Pro Asn Lys Tyr Ser Thr Lys
1155 1160 1165

Val Gly Asp Lys Gly Thr Gln Leu Ser Gly Gly Gln Lys Gln Arg Ile
1170 1175 1180

Ala Ile Ala Arg Ala Leu Val Arg Gln Pro His Ile Leu Leu Leu Asp
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Glu Ala Thr Ser Ala Leu Asp Thr Glu Ser Glu Lys Val Val Gln Glu
1205 1210 1215

Ala Leu Asp Lys Ala Arg Glu Gly Arg Thr Cys Ile Val Ile Ala His
1220 1225 1230

Arg Leu Ser Thr Ile Gln Asn Ala Asp Leu Ile Val Val Phe Gln Asn
1235 1240 1245

Gly Arg Val Lys Glu His Gly Thr His Gln Gln Leu Leu Ala Gln Lys
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<211> 4189

<212> DNA

<213> MOUSE MDR-1

<400> 5

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<211> 1276

<212> PRT

<213> MOUSE MDR-1

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Val Gly Val Phe Gly Met Phe Arg Tyr Ala Asp Trp Leu Asp Lys Leu
35 40 45

Cys Met Ile Leu Gly Thr Leu Ala Ala Ile Ile His Gly Thr Leu Leu
50 55 60

Pro Leu Leu Met Leu Val Phe Gly Asn Met Thr Asp Ser Phe Thr Lys
65 70 75 80

Ala Glu Ala Ser Ile Leu Pro Ser Ile Thr Asn Gln Ser Gly Pro Asn
85 90 95

Ser Thr Leu Ile Ile Ser Asn Ser Ser Leu Glu Glu Glu Met Ala Ile
100 105 110

Tyr Ala Tyr Tyr Tyr Thr Gly Ile Gly Ala Gly Val Leu Ile Val Ala
115 120 125

Tyr Ile Gln Val Ser Leu Trp Cys Leu Ala Ala Gly Arg Gln Ile His
130 135 140

Lys Ile Arg Gln Lys Phe Phe His Ala Ile Met Asn Gln Glu Ile Gly
145 150 155 160

Trp Phe Asp Val His Asp Val Gly Glu Leu Asn Thr Arg Leu Thr Asp
165 170 175

Asp Val Ser Lys Ile Asn Asp Gly Ile Gly Asp Lys Ile Gly Met Phe
180 185 190

Phe Gln Ser Ile Thr Thr Phe Leu Ala Gly Phe Ile Ile Gly Phe Ile
195 200 205

Ser Gly Trp Lys Leu Thr Leu Val Ile Leu Ala Val Ser Pro Leu Ile

210 215 220

Gly Leu Ser Ser Ala Leu Trp Ala Lys Val Leu Thr Ser Phe Thr Asn
225 230 235 240

Lys Glu Leu Gln Ala Tyr Ala Lys Ala Gly Ala Val Ala Glu Glu Val
245 250 255

Leu Ala Ala Ile Arg Thr Val Ile Ala Phe Gly Gly Gln Gln Lys Glu
260 265 270

Leu Glu Arg Tyr Asn Lys Asn Leu Glu Glu Ala Lys Asn Val Gly Ile
275 280 285

Lys Lys Ala Ile Thr Ala Ser Ile Ser Ile Gly Ile Ala Tyr Leu Leu
290 295 300

Val Tyr Ala Ser Tyr Ala Leu Ala Phe Trp Tyr Gly Thr Ser Leu Val
305 310 315 320

Leu Ser Asn Glu Tyr Ser Ile Gly Glu Val Leu Thr Val Phe Phe Ser
325 330 335

Ile Leu Leu Gly Thr Phe Ser Ile Gly His Leu Ala Pro Asn Ile Glu
340 345 350

Ala Phe Ala Asn Ala Arg Gly Ala Ala Phe Glu Ile Phe Lys Ile Ile
355 360 365

Asp Asn Glu Pro Ser Ile Asp Ser Phe Ser Thr Lys Gly Tyr Lys Pro
370 375 380

Asp Ser Ile Met Gly Asn Leu Glu Phe Lys Asn Val His Phe Asn Tyr
385 390 395 400

Pro Ser Arg Ser Glu Val Gln Ile Leu Lys Gly Leu Asn Leu Lys Val
405 410 415

Lys Ser Gly Gln Thr Val Ala Leu Val Gly Asn Ser Gly Cys Gly Lys
420 425 430

Ser Thr Thr Val Gln Leu Met Gln Arg Leu Tyr Asp Pro Leu Glu Gly
435 440 445

Val Val Ser Ile Asp Gly Gln Asp Ile Arg Thr Ile Asn Val Arg Tyr
450 455 460

Leu Arg Glu Ile Ile Gly Val Val Ser Gln Glu Pro Val Leu Phe Ala
465 470 475 480

Thr Thr Ile Ala Glu Asn Ile Arg Tyr Gly Arg Glu Asp Val Thr Met
485 490 495

Asp Glu Ile Glu Lys Ala Val Lys Glu Ala Asn Ala Tyr Asp Phe Ile
500 505 510

Met Lys Leu Pro His Gln Phe Asp Thr Leu Val Gly Glu Arg Gly Ala
515 520 525

Gln Leu Ser Gly Gly Gln Lys Gln Arg Ile Ala Ile Ala Arg Ala Leu
530 535 540

Val Arg Asn Pro Lys Ile Leu Leu Leu Asp Glu Ala Thr Ser Ala Leu
545 550 555 560

Asp Thr Glu Ser Glu Ala Val Val Gln Ala Ala Leu Asp Lys Ala Arg
565 570 575

Glu Gly Arg Thr Thr Ile Val Ile Ala His Arg Leu Ser Thr Val Arg
580 585 590

Asn Ala Asp Val Ile Ala Gly Phe Asp Gly Gly Val Ile Val Glu Gln
595 600 605

Gly Asn His Asp Glu Leu Met Arg Glu Lys Gly Ile Tyr Phe Lys Leu
610 615 620

Val Met Thr Gln Thr Arg Gly Asn Glu Ile Glu Pro Gly Asn Asn Ala
625 630 635 640

Tyr Gly Ser Gln Ser Asp Thr Asp Ala Ser Glu Leu Thr Ser Glu Glu
645 650 655

Ser Lys Ser Pro Leu Ile Arg Arg Ser Ile Tyr Arg Ser Val His Arg
660 665 670

Lys Gln Asp Gln Glu Arg Arg Leu Ser Met Lys Glu Ala Val Asp Glu

675 680 685

Asp Val Pro Leu Val Ser Phe Trp Arg Ile Leu Asn Leu Asn Leu Ser
690 695 700

Glu Trp Pro Tyr Leu Leu Val Gly Val Leu Cys Ala Val Ile Asn Gly
705 710 715 720

Cys Ile Gln Pro Val Phe Ala Ile Val Phe Ser Arg Ile Val Gly Val
725 730 735

Phe Ser Arg Asp Asp Asp His Glu Thr Lys Arg Gln Asn Cys Asn Leu
740 745 750

Phe Ser Leu Phe Phe Leu Val Met Gly Leu Ile Ser Phe Val Thr Tyr
755 760 765

Phe Phe Gln Gly Phe Thr Phe Gly Lys Ala Gly Glu Ile Leu Thr Lys
770 775 780

Arg Val Arg Tyr Met Val Phe Lys Ser Met Leu Arg Gln Asp Ile Ser
785 790 795 800

Trp Phe Asp Asp His Lys Asn Ser Thr Gly Ser Leu Thr Thr Arg Leu
805 810 815

Ala Ser Asp Ala Ser Ser Val Lys Gly Ala Met Gly Ala Arg Leu Ala
820 825 830

Val Val Thr Gln Asn Val Ala Asn Leu Gly Thr Gly Val Ile Leu Ser
835 840 845

Leu Val Tyr Gly Trp Gln Leu Thr Leu Leu Leu Val Val Ile Ile Pro
850 855 860

Leu Ile Val Leu Gly Gly Ile Ile Glu Met Lys Leu Leu Ser Gly Gln
865 870 875 880

Ala Leu Lys Asp Lys Lys Gln Leu Glu Ile Ser Gly Lys Ile Ala Thr
885 890 895

Glu Ala Ile Glu Asn Phe Arg Thr Ile Val Ser Leu Thr Arg Glu Gln
900 905 910

Lys Phe Glu Thr Met Tyr Ala Gln Ser Leu Gln Val Pro Tyr Arg Asn
915 920 925

Ala Met Lys Lys Ala His Val Phe Gly Ile Thr Phe Ser Phe Thr Gln
930 935 940

Ala Met Met Tyr Phe Ser Tyr Ala Ala Cys Phe Arg Phe Gly Ala Tyr
945 950 955 960

Leu Val Ala Gln Gln Leu Met Thr Phe Glu Asn Val Met Leu Val Phe
965 970 975

Ser Ala Val Val Phe Gly Ala Met Ala Ala Gly Asn Thr Ser Ser Phe
980 985 990

Ala Pro Asp Tyr Ala Lys Ala Lys Val Ser Ala Ser His Ile Ile Arg
995 1000 1005

Ile Ile Glu Lys Thr Pro Glu Ile Asp Ser Tyr Ser Thr Glu Gly Leu
1010 1015 1020

Lys Pro Thr Leu Leu Glu Gly Asn Val Lys Phe Asn Gly Val Gln Phe
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Asn Tyr Pro Thr Arg Pro Asn Ile Pro Val Leu Gln Gly Leu Ser Leu
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Glu Val Lys Lys Gly Gln Thr Leu Ala Leu Val Gly Ser Ser Gly Cys
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Gly Lys Ser Thr Val Val Gln Leu Leu Glu Arg Phe Tyr Asp Pro Met
1075 1080 1085

Ala Gly Ser Val Phe Leu Asp Gly Lys Glu Ile Lys Gln Leu Asn Val
1090 1095 1100

Gln Trp Leu Arg Ala His Leu Gly Ile Val Ser Gln Glu Pro Ile Leu
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Phe Asp Cys Ser Ile Ala Glu Asn Ile Ala Tyr Gly Asp Asn Ser Arg
1125 1130 1135

Ala Val Ser His Glu Glu Ile Val Arg Ala Ala Lys Glu Ala Asn Ile

1140 1145 1150

His Gln Phe Ile Asp Ser Leu Pro Asp Lys Tyr Asn Thr Arg Val Gly
1155 1160 1165

Asp Lys Gly Thr Gln Leu Ser Gly Gly Gln Lys Gln Arg Ile Ala Ile
1170 1175 1180

Ala Arg Ala Leu Val Arg Gln Pro His Ile Leu Leu Leu Asp Glu Ala
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Thr Ser Ala Leu Asp Thr Glu Ser Glu Lys Val Val Gln Glu Ala Leu
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Asp Lys Ala Arg Glu Gly Arg Thr Cys Ile Val Ile Ala His Arg Leu
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Ser Thr Ile Gln Asn Ala Asp Leu Ile Val Val Ile Glu Asn Gly Lys
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Tyr Met Leu Val Gly Thr Leu Ala Ala Ile Ile His Gly Val Ala Leu
50 55 60

Pro Leu Met Met Leu Ile Phe Gly Asp Met Thr Asp Ser Phe Ala Ser
65 70 75 80

Val Gly Asn Val Ser Lys Asn Ser Thr Asn Met Ser Glu Ala Asp Lys
85 90 95

Arg Ala Met Phe Ala Lys Leu Glu Glu Glu Met Thr Thr Tyr Ala Tyr
100 105 110

Tyr Tyr Thr Gly Ile Gly Ala Gly Val Leu Ile Val Ala Tyr Ile Gln
115 120 125

Val Ser Phe Trp Cys Leu Ala Ala Gly Arg Gln Ile His Lys Ile Arg

130 135 140

Gln Lys Phe Phe His Ala Ile Met Asn Gln Glu Ile Gly Trp Phe Asp
145 150 155 160

Val His Asp Val Gly Glu Leu Asn Thr Arg Leu Thr Asp Asp Val Ser
165 170 175

Lys Ile Asn Glu Gly Ile Gly Asp Lys Ile Gly Met Phe Phe Gln Ala
180 185 190

Met Ala Thr Phe Phe Gly Gly Phe Ile Ile Gly Phe Thr Arg Gly Trp
195 200 205

Lys Leu Thr Leu Val Ile Leu Ala Ile Ser Pro Val Leu Gly Leu Ser
210 215 220

Ala Gly Ile Trp Ala Lys Ile Leu Ser Ser Phe Thr Asp Lys Glu Leu
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His Ala Tyr Ala Lys Ala Gly Ala Val Ala Glu Glu Val Leu Ala Ala
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Ile Arg Thr Val Ile Ala Phe Gly Gly Gln Lys Lys Glu Leu Glu Arg
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Tyr Asn Asn Asn Leu Glu Glu Ala Lys Arg Leu Gly Ile Lys Lys Ala
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Ile Thr Ala Asn Ile Ser Met Gly Ala Ala Phe Leu Leu Ile Tyr Ala
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Ser Tyr Ala Leu Ala Phe Trp Tyr Gly Thr Ser Leu Val Ile Ser Lys
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Glu Tyr Ser Ile Gly Gln Val Leu Thr Val Phe Phe Ser Val Leu Ile
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Gly Ala Phe Ser Val Gly Gln Ala Ser Pro Asn Ile Glu Ala Phe Ala
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Asn Ala Arg Gly Ala Ala Tyr Glu Val Phe Lys Ile Ile Asp Asn Lys
355 360 365

Pro Ser Ile Asp Ser Phe Ser Lys Ser Gly His Lys Pro Asp Asn Ile
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Gln Gly Asn Leu Glu Phe Lys Asn Ile His Phe Ser Tyr Pro Ser Arg
385 390 395 400

Lys Glu Val Gln Ile Leu Lys Gly Leu Asn Leu Lys Val Lys Ser Gly
405 410 415

Gln Thr Val Ala Leu Val Gly Asn Ser Gly Cys Gly Lys Ser Thr Thr
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Val Gln Leu Met Gln Arg Leu Tyr Asp Pro Leu Asp Gly Met Val Ser
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Ile Ile Gly Val Val Ser Gln Glu Pro Val Leu Phe Ala Thr Thr Ile
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Ala Glu Asn Ile Arg Tyr Gly Arg Glu Asp Val Thr Met Asp Glu Ile
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Glu Lys Ala Val Lys Glu Ala Asn Ala Tyr Asp Phe Ile Met Lys Leu
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Pro His Gln Phe Asp Thr Leu Val Gly Glu Arg Gly Ala His Val Ser
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Gly Gly Gln Lys Gln Arg Ile Ala Ile Ala Arg Ala Leu Val Arg Asn
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545 550 555 560

Ser Glu Ala Val Val Gln Ala Ala Leu Asp Lys Ala Arg Glu Gly Arg
565 570 575

Thr Thr Ile Val Ile Ala His Arg Leu Ser Thr Val Arg Asn Ala Asp
580 585 590

Val Ile Ala Gly Phe Asp Gly Gly Val Ile Val Glu Gln Gly Asn His

595 600 605

Asp Glu Leu Met Arg Glu Lys Gly Ile Tyr Phe Lys Leu Val Met Thr
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Gln Thr Ala Gly Asn Glu Ile Glu Leu Gly Asn Glu Ala Cys Lys Ser
625 630 635 640

Lys Asp Glu Ile Asp Asn Leu Asp Met Ser Ser Lys Asp Ser Gly Ser
645 650 655

Ser Leu Ile Arg Arg Arg Ser Thr Arg Lys Ser Ile Cys Gly Pro His
660 665 670

Asp Gln Asp Arg Lys Leu Ser Thr Lys Glu Ala Leu Asp Glu Asp Val
675 680 685

Pro Pro Ala Ser Phe Trp Arg Ile Leu Lys Leu Asn Ser Thr Glu Trp
690 695 700

Pro Tyr Phe Val Val Gly Ile Phe Cys Ala Ile Ile Asn Gly Gly Leu
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Gln Pro Ala Phe Ser Val Ile Phe Ser Lys Val Val Gly Val Phe Thr
725 730 735

Asn Gly Gly Pro Pro Glu Thr Gln Arg Gln Asn Ser Asn Leu Phe Ser
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Leu Leu Phe Leu Ile Leu Gly Ile Ile Ser Phe Ile Thr Phe Phe Leu
755 760 765

Gln Gly Phe Thr Phe Gly Lys Ala Gly Glu Ile Leu Thr Lys Arg Leu
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Arg Tyr Met Val Phe Lys Ser Met Leu Arg Gln Asp Val Ser Trp Phe
785 790 795 800

Asp Asp Pro Lys Asn Thr Thr Gly Ala Leu Thr Thr Arg Leu Ala Asn
805 810 815

Asp Ala Ala Gln Val Lys Gly Ala Thr Gly Ser Arg Leu Ala Val Ile
820 825 830

Phe Gln Asn Ile Ala Asn Leu Gly Thr Gly Ile Ile Ile Ser Leu Ile
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Tyr Gly Trp Gln Leu Thr Leu Leu Leu Leu Ala Ile Val Pro Ile Ile
850 855 860

Ala Ile Ala Gly Val Val Glu Met Lys Met Leu Ser Gly Gln Ala Leu
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Lys Asp Lys Lys Glu Leu Glu Gly Ser Gly Lys Ile Ala Thr Glu Ala
885 890 895

Ile Glu Asn Phe Arg Thr Val Val Ser Leu Thr Arg Glu Gln Lys Phe
900 905 910

Glu Thr Met Tyr Ala Gln Ser Leu Gln Ile Pro Tyr Arg Asn Ala Met
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Lys Lys Ala His Val Phe Gly Ile Thr Phe Ser Phe Thr Gln Ala Met
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Met Tyr Phe Ser Tyr Ala Ala Cys Phe Arg Phe Gly Ala Tyr Leu Val
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Ile Val Phe Gly Ala Met Ala Val Gly Gln Val Ser Ser Phe Ala Pro
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Asp Tyr Ala Lys Ala Thr Val Ser Ala Ser His Ile Ile Arg Ile Ile
995 1000 1005

Glu Lys Thr Pro Glu Ile Asp Ser Tyr Ser Thr Gln Gly Leu Lys Pro
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Asn Met Leu Glu Gly Asn Val Gln Phe Ser Gly Val Val Phe Asn Tyr
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Pro Thr Arg Pro Ser Ile Pro Val Leu Gln Gly Leu Ser Leu Glu Val
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Phe Ile Asp Ser Leu Pro Asp Lys Tyr Asn Thr Arg Val Gly Asp Lys
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